8-ALKOXYADENOSINES MODULATE RNA INTERFERENCE EFFICACY AND OFF-PATHWAY PROTEIN BINDING

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

Despite the tremendous potential of short interfering RNA (siRNA) as a novel biopharmaceutical, its therapeutic utility has not been maximized mainly due to lack of proper *in vivo* delivery vehicle, off-target effects and several off-pathway protein interactions instigating immunostimulation. Judicious chemical modification of different parts of the siRNA was foreseen as a potential solution to the off-target gene silencing and the off-pathway protein binding.

In this study, 8-alkoxyadenosines were explored as a nucleobase modification in the context of the siRNA-based RNA interference (RNAi). These nucleosides are unusual in that they have the potential to exist as an equilibrium mixture of the *syn* and *anti* conformers. When placed opposite to U in the complementary strand, 8-alkoxyadenosines can exist in normal *anti* conformation and form canonical Watson-Crick hydrogen bonding; interestingly, with G as the base-pairing partner, these unusual nucleosides can potentially flip into the *syn* conformation and form unorthodox Hoogsteen base-pairing.

8-Alkoxyadenosine phosphoramidites were synthesized and incorporated into the guide strand of caspase 2 siRNA at four different positions - two in the seed region, one at the cleavage junction and another nearer to the 3´-end of the guide strands. Thermal stabilities of the corresponding siRNA duplexes showed that U is still preferred over G as the base-pairing partner in the complementary strand. When compared to the unmodified

positive control siRNAs, singly modified siRNAs have knocked down caspase 2 insert mRNA (generated from a recombinant plasmid) efficiently and with little or no loss of efficacy. Doubly modified siRNAs were found to be less effective and lose their efficacy at low nanomolar concentrations. Persistent placement of steric blockade in the minor groove affected the RNAi efficacy significantly; this observation supports the hypothesis and indicates the necessity of 'switching' the bulky alkyloxy groups in the major groove, when modified siRNAs interact with the RISC assembly.

SiRNAs modified at positions 6 and 10 of the guide strand were found to be effective against preventing interaction with the RNA-dependent protein kinase (PKR). In summary, 8-alkoxyadenosine-containing siRNAs prevented undesired off-pathway protein binding, without compromising the RNAi efficacy significantly.

For my parents

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LIST OF ABBREVIATIONS

A	adenosine
ADAR	adenosine deaminases that act on RNA
Ago	argonaute
AMD	age-related macular degeneration
Arg	arginine
8-BrA	8-bromoadenosine
8-BrdA	2′-deoxy-8-bromoadenosine
C	cytosine
8-CeOA	8-cyclohexylethyloxyadenosine
8-ClA	8-chloroadenosine
dA	2′-deoxyadenosine
dC	2′-deoxycytosine
dG	2′-deoxyguanosine
DMEM	Dulbecco's Modified Eagle's Medium
DNA	2′-deoxyribonucleic acid
dsRB	double-stranded RNA-binding
dsRBMs	double stranded RNA-binding motifs
dsRNA	double-stranded RNA
dT	2′-deoxythymine

EBER	Epstein-Barr virus-associated encoding small RNA
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
eIF2!	eukaryotic initiation factor 2 alpha subunit
ESI-MS	electrospray ionization mass spectrometry
FBS	fetal bovine serum
G	guanosine
HRMS	high resolution mass spectrometry
HPLC	high performance liquid chromatography
INF	Interferon
LNA	locked nucleic acid
miRNA	microRNA
mRNA	messenger RNA
NOESY	nuclear overhauser effect spectroscopy
NMR	nuclear magnetic resonance
OAS	2′, 5′-oligoadenylate synthase
8-OxodG	2′-deoxy-7,8-dihydro-8-oxoguanosine
PAGE	polyacrylamide gel electrophoresis
PAZ	PIWI/Argonaute/Zwille domain
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PDC	plasmacytoid dendritic cells
8-PeOA	8-phenethyloxyadenosine

8-PgOA	8-propargyloxyadenosine
PIWI	P-element-induced whimpy testes domain
PKR	RNA-dependent protein kinase
PNA	peptide nucleic acid
RBP	RNA-binding protein
RISC	RNA-Induced Silencing Complex
RLC	RISC loading complex
RNA	ribonucleic acid
8-RNHA	8-alkylaminoadenosine
8-ROA	8-alkoxyadenosine
RSV	respiratory syncytial virus
rtPCR	real-time PCR
siRNA	short interfering RNA
ssRNA	single-stranded DNA
STAT	signal transducers/activators
TAR	HIV-1 transactivation responsive element
TLC	thin layer chromatography
TLR	Toll-like receptor
TRAP	trp RNA-binding attenuation protein
TRBP	HIV-1 transactivation responsive element
SNP	single nucleotide polymorphism
UNA	unlocked nucleic acid
UTR	untranslated region

WC	Watson-Crick
T	thymine
T_m	melting temperature
TMS	tetramethylsilane
Tris	tris(hydroxymethyl)-aminomethane
U	uracil
UV	ultraviolet
Vis	visible

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CHAPTER 1

SHORT INTERFERING RNA THERAPEUTICS AND CHEMICAL MODIFICATIONS

RNA interference and short interfering RNA

RNA interference (RNAi) has become a crucial tool to interrogate and analyze gene function since the discovery of double stranded RNA (dsRNA)-mediated RNAi in *C. elegans* in 1998 (*1*). Theoretically, any mRNA *in vitro* or *in vivo* can selectively be knocked down by short RNA oligomers containing 19-23 nucleotides called short interfering RNAs (siRNAs). In 2001, Elbashir et al. showed that RNAi was effective in mammalian cells, and the therapeutic potential of siRNA was quickly understood (*2*). This finding triggered a plethora of research on utilization of siRNA as therapeutics (*3-6*).

SiRNA-mediated gene silencing is highly selective provided siRNAs are rationally chosen. Thermodynamic stability of the duplex is an important determining factor. The duplex should be less stable towards the 5′-end of the guide strand compared to the 3′-end. Nucleotides 2-8 from the 5′-end of the guide strand, also called the seed region of an siRNA (Figure 1.1), play crucial roles in mRNA knockdown efficiency and specificity. Nucleotides 10 and 11 are the cleavage site nucleotides; the mRNA cleavage occurs between these two positions. Generally, there should be perfect base pairing in the seed region and at the cleavage site for optimal efficacy; sometimes, mismatches (*7, 8*) are tolerated at the expense of RNAi efficacy.

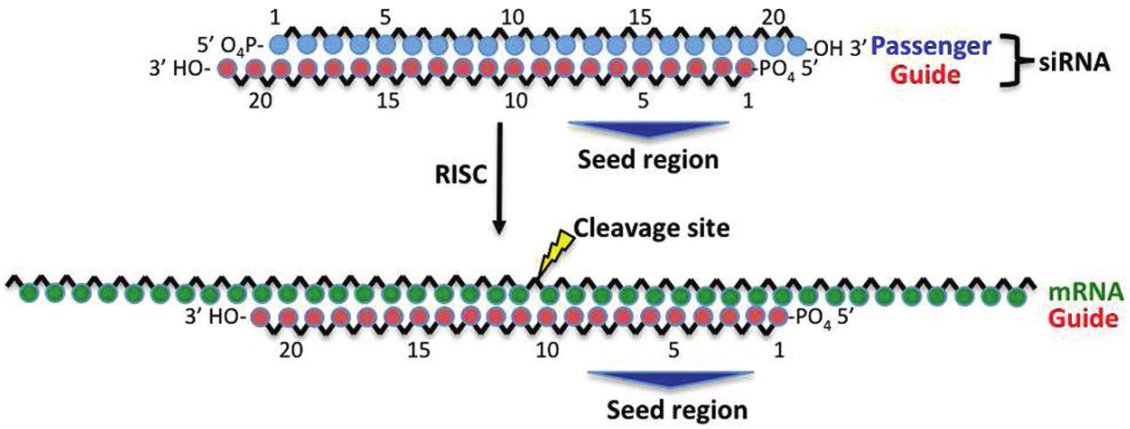

Figure 1.1. Structure of an siRNA. (reproduced with permission from reference (*9*)).

SiRNAs can be generated in the cell from long dsRNAs (by Dicer) or can be chemically synthesized and delivered inside cells to mediate specific mRNA cleavage (Figure 1.2). Upon entry into the cellular environment, siRNA is phosphorylated at the 5′-end of the strands and loaded into the RNA-induced silencing complex (RISC). In the RISC, enzyme helicase unwind the siRNA and the active siRNP complex is generated where the guide strand mediates the cleavage of complementary mRNA.

RNAi: Proof-of-principle in animal models

Local RNAi in animal models. To date, most of the siRNAs have been successfully applied through local administration. Specific silencing of target genes has been achieved in animal models of human diseases using appropriate siRNA or shRNA (short hairpin RNA). The RNAi concept has been validated in multiple organs such as, lung (*10, 11*), brain (*12-20*), colon (*21, 22*), eye (*23-25*), and vagina(*26*) and diseases including viral infection (*10, 11, 27, 28*), nervous diseases (*12-16, 29, 30*), cancers (*22, 31-36*), irritable bowel syndrome(*21*), etc.(*37*) (Figure 1.3).

Highly promising results have been obtained in rodent (mouse) and primate (monkey) models of severe acute respiratory syndrome (SARS) (*11*), RSV (respiratory syncitical virus) and influenza. The cationic polymer-based transfection reagent TransIT TKO has successfully led to intranasal delivery of siRNAs against RSV and para-influenza virus into pulmonary tissues and reduced the infection by more than 99% (*10*). Lethal Ebola virus infection was successfully countered by administration of SNALP-formulated siRNAs in guinea pigs (*28*).

RNAi proof-of-concept studies have been demonstrated in numerous ocular diseases, in animal models, through local intravitreal delivery of specific siRNA. SiRNAs

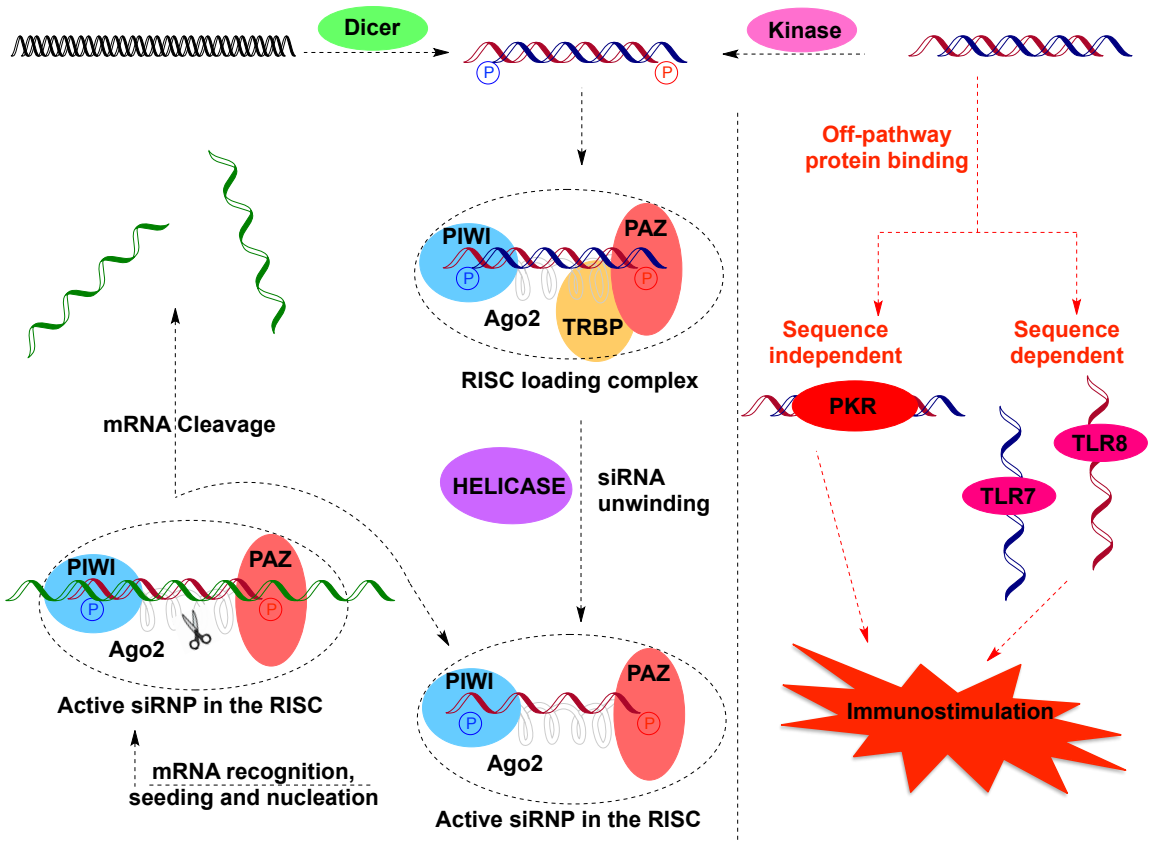

Figure 1.2. Mechanism and side effects of the siRNA-based RNAi.

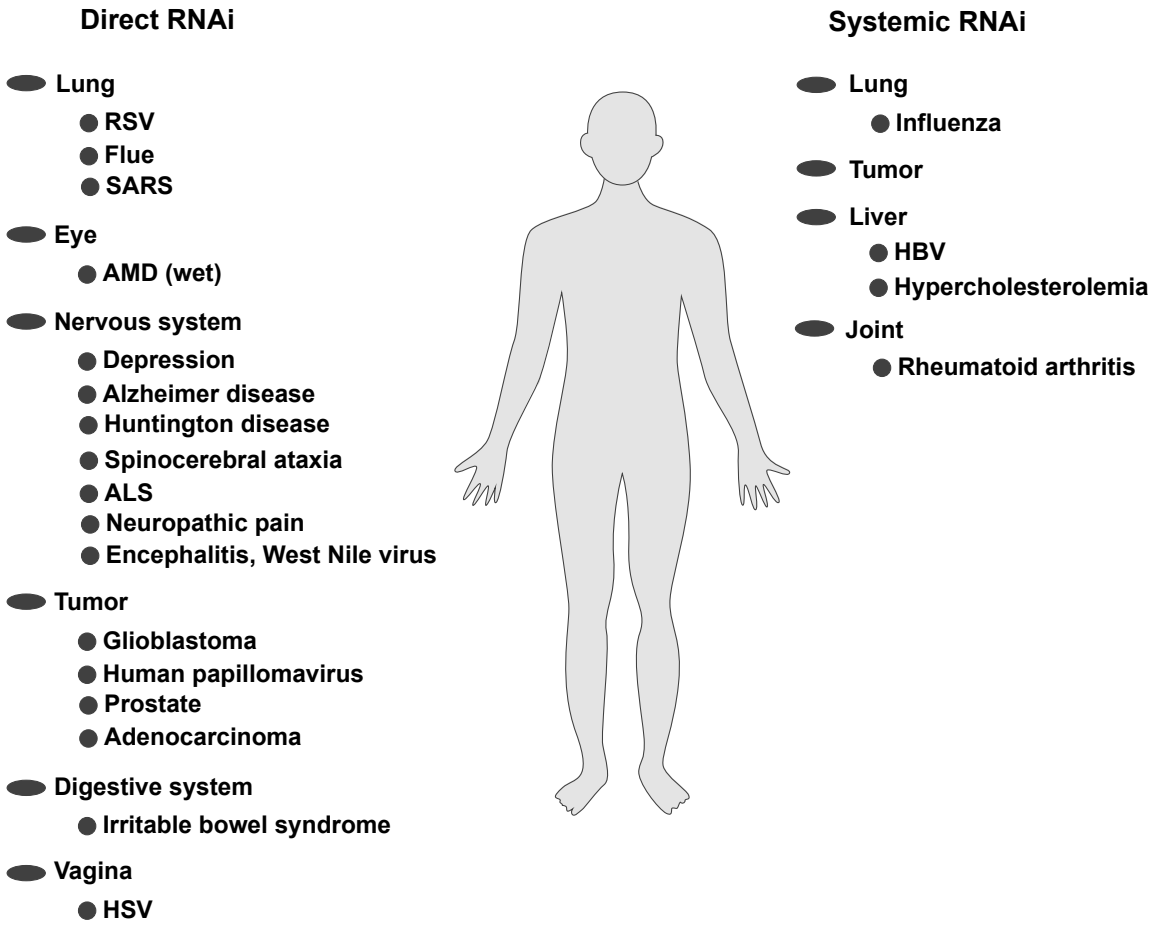

Figure 1.3. Organs and diseases where siRNA-based RNAi has been demonstrated. (redrawn with permission from reference (*37*).

targeting vascular endothelial growth factor (VEGF) receptor-1 and transforming growth factor (TGF)-β receptor type II were effective in shrinking ocular neovascularization and preventing choroidal neovascularization respectively in two separate mouse models (*23, 24*). For local siRNA delivery in the eye, saline and lipid formulations were proven effective. Mucosal membranes in colon, nose and genitals are accessible to various siRNA formulations, e.g., lipofectamine formulated siRNAs targeting TNF-α in IBS has been shown to reduce TNF-α abundance, as well as reduce inflammation of the colon (*21*).

ShRNA- or siRNA-based RNAi was shown to be effective *in vivo* in numerous nervous disease models (*12-16, 29, 30*). RNAi has brought about marked reduction in disease phenotypes and neuropathology (*16*) in the xenograft models of Alzheimer's disease (*16*), Huntington's disease (*13*), amyotrophic lateral sclerosis (ALS) (*14, 15*), chronic neuropathic pain (*38*), cerebellar ataxia (*12*), encephalitis (*30*), etc. In these studies on specific neurons, even 10-20% reduction of the target gene expression has been reported to normalize disease symptoms significantly (*16*).

The RNAi concept has also been validated in different mouse xenograft models of human cancers through appropriate delivery vehicles (*37*), such as lipids (*31, 39, 40*), polymers (*41*), cholesterol-oligoarginine (*22*), protamine-Fab fusion protein (*42*), and aptamers (*43*). Aptamer-based chimeric siRNAs promise to be the most simplified and selective approach, provided an aptamer is available for a tumor specific receptor. Aptamer-siRNA conjugates have successfully prevented tumor growth in xenograft models of prostate cancer (*34*).

Systemic RNAi in animal models. In systemic administration of siRNA, RNAi efficacy is dependent on the choice of delivery vehicles and chemical modifications. Systemic RNAi (*44, 45*) has been demonstrated in mouse models of several diseases such as hypercholesterolemia (*46*), rheumatoid arthritis, viral infections (hepatitis B virus (*27*) , influenza virus, Ebola virus (*28*)) and in tumor xenografts. Cholesterol-conjugated and SNALP (stable nucleic acid lipid particle)-formulated siRNAs have been shown to silence apolipoprotein apoB in nonhuman primates (*47*).

A spectrum of delivery vehicles has successfully delivered anticancer siRNAs into various malignant tumors. Cationic cardiolipin liposome formulated siRNAs, when delivered intravenously, prevented tumor growth in xenograft model of prostate cancer (*48*). Systemic administration of PEGylated PEI-Arg-Gly-Asp nanoparticle formulated siRNAs targeting VEGF R-2 (vascular endothelial growth factor receptor-2) has prevented tumor angiogenesis and growth (*49*). Cholesterol-conjugated siRNAs targeting VEGF receptor inhibited tumor growth in colon adenocarcinoma (*22*). Intravenous delivery of atelo-collagen-siRNA complexes have pronounced effects on bone tumors (*35*) and subcutaneous tumor xenografts (*50*). Ligand-directed systemic delivery of siRNA is gradually gaining importance; siRNA complexed with transferrin-anchored polymeric nanoparticles has shrunk metastatic Ewing sarcoma (*36*).

SiRNA clinical trials

In spite of the challenges of finding appropriate delivery vehicles for siRNA, at least eleven siRNAs are currently in clinical trials (Table 1.1). Validation of the siRNA-based RNAi in xenograft models of human diseases has encouraged clinical trials of siRNAs in the treatment of several diseases (*3, 51, 52*). Prior success with antisense oligo

Table 1.1 SiRNA clinical trials. (reproduced with permission from reference (*6*))

Company	Drug name	Phase	Target gene and disease	Chemically modified?	Delivery (vehicle, if known)
Alnylam	ALN-RSV01	II	RSV (viral nucleocapsid)	No	Intranasal or inhaled
Quark/Pfizer	PF-04523655	II	RTP801 for wet AMD or diabetic macular edema	Yes	Intraocular
Quark	QPI-1002	II	p53 for acute renal failure	Yes	Intravenous
ZaBeCor	Excellair	II	SYK kinase for asthma	Unknown	Inhaled
Calando	CALAA-01	I	Ribonucleotide reductase (RRM2) for solid tumors	No	Intravenous (targeted CD nanoparticle)
TransDerm	TD101	Ib	Keratin 6a (N171K mutation) for Pachyonychia Congenita	Unknown	Intradermal injection
Alnylam	ALN-VSP01	I	VEGF and KSP for liver cancer	Yes	Intravenous (lipid nanoparticle)
Silence (Atugen)	Atu-027	I	PKN3 for solid tumors—specifically tumor vasculature	Yes	Intravenous (lipid nanoparticle)
Tekmira	ApoB SNALP	I	ApoB for hypercholesterolemia	Yes	Intravenous (lipid nanoparticle)
Sylentis	SYL-040012	I	ADRB2 for ocular hypertension (glaucoma)	Unknown	Intraocular
Quark	QPI-1007	I	Caspase-2 for glaucoma or acute eye injury	Yes	Intraocular
Drugs that have been withdrawn from clinical trials					
OPKO (acuity)	Bevasiranib	III	All VEGF-A isoforms for wet AMD	No	Intraocular
Sirna/Merck with allergan	AGN-211745	II	VEGF receptor (VEGF-R1) for wet AMD	Yes	Intraocular

nucleotides, led to initial clinical trials of siRNAs targeting ocular diseases such as AMD and diabetic macular edema (DME). In both the cases VEGF pathway was targeted locally with intraocular injection of siRNA. After initial clinical trials both were withdrawn from the market due to safety reasons and availability of alternative therapeutics (*53*). Still, the eye is an attractive target for RNAi validation (*54, 55*). SiRNA (PF-04523655) targeting hypoxia-responsive gene RTP801 to treat AMD and DME is already in advanced clinical trial (phase II) (*55*); visual acuity is restored in 90% of the patients in a preliminary study. Two other drugs for the treatment of glaucoma are currently under phase I clinical trial (*6*).

Due to high efficacy and specificity of siRNAs against RSV in mice models, Alnylam has designed ALN-RSV01 siRNA, which is currently in phase II clinical trial (*56*). Two siRNAs, for acute renal failure and asthma, are under phase II clinical trial at the moment (*6*). There has been significant advancement in the development of siRNAs targeting cancer. Several targets for siRNA therapeutics (under phase I clinical trial) in this category are ribonucleotide reductase (RRM2) for solid tumors, PKN3 for tumor vasculature in solid tumors, VEGF and KSP for liver cancer etc. Tekmira is developing lipid nanoparticle-formulated siRNAs targeting ApoB for hypercholesterolemia (*6*). All of them are being developed for systemic administration. Transderm is developing siRNAs against pachyonychia congenita, a keratin 6a abnormality; they have successfully targeted a single-point mutant mRNA corresponding to keratin 6a (*57, 58*).

SiRNA delivery

SiRNA delivery techniques have improved significantly over the last few years (Figure 1.4) (*59*). Polymeric or liposomal formulations reduced the dose requirement (per

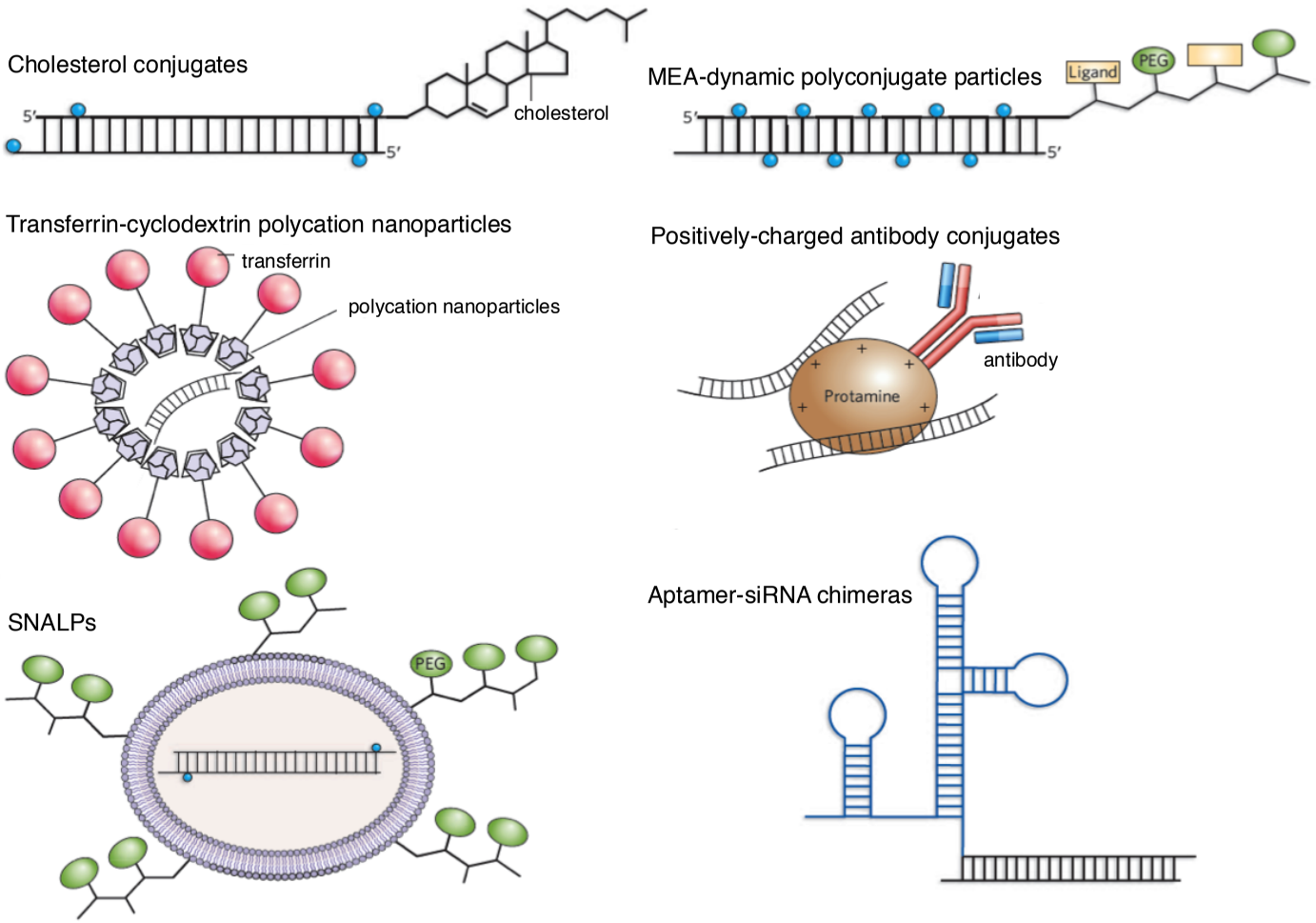

Figure 1.4. *In vivo* delivery vehicles of the siRNA. (adapted with permission from reference (*3*))

day) of naked siRNA in efficacious RNAi; compared to 0.4 mg of siRNA formulated in PBS, only 5-40 μg is required if formulated in appropriate polymeric or liposomal delivery vehicles. Reduction in dosage also helps reduce undesired off-target effects and immunostimulation. Also in ocular clinical trials, it was found that only cholesterol-conjugated siRNAs can enter the RNAi pathway and naked siRNAs merely interact with the innate immune system (*60*). These observations clearly suggest that appropriate delivery vehicles are essential for therapeutic efficacy of siRNA.

Polymeric nanocarriers are gradually gaining importance as siRNA delivery vehicles (*41, 61-64*). PEG-based and pH sensitive cationic copolymers (*65*), lactose-conjugated pH-sensitive PEG (*66*) and chitosan-polyethylene oxide copolymer (*67*) have been shown to enhance intracellular gene silencing. Acid-lability and bioreduciblity of polymer backbones have been judiciously used for the controlled release of siRNA in desired targets (*62, 66, 68*). Recently, phosphonium ion-based cationic polymers were employed as a nontoxic alternative to the polyammonium carriers (*69*). Several macroscopic biomaterial scaffolds, such as PEG, alginate, photoalginate, collagen etc, have been reported for localized, targeted and sustained delivery of siRNA (*63, 70*). Proton sponge-anchored quantum dots have been used both to deliver siRNA to appropriate target and image the entire delivery and trafficking process inside cell (*71, 72*).

Lipid-based delivery vehicles are also being successfully used for *in vivo* RNAi studies (Table 1.2) (*39, 40, 73*). SiRNAs-conjugated to lipid-like molecules have successfully delivered siRNAs in cultured cells and *in vivo*. These covalently conjugated lipidoids are promising for both local and systemic delivery of siRNA (*74*).

Table 1.2. *In vivo* delivery strategies for therapeutic siRNAs. (reproduced with permission from reference (*75*))

Material	Model	Target	Route	Animal
Liposomes and lipids				
i-FECT	Japanese encephalitis virus (JEV) and West Nile virus (WNV)	JEV and WNV envelope	Intracranial	Mouse
Lipidoids	Dyslipidaemia	FVII/ApoB	Intravenous	Mouse, rat, monkey
	Dyslipidaemia	FVII/ApoB	Intravenous	Mouse, hamster
	Malaria	Haem oxygenase 1	Intravenous	Mouse
	Hypercholesterolaemia	PCSK9	Intravenous	Mouse, rat
LipoTrust	Liver cirrhosis	gp46	Intravenous	Rat
Oligofectamine	Herpes simplex virus 2 (HSV-2)	HSV-2-associated viral proteins UL27 and UL29	Intravaginal	Mouse
SNALP	Hepatitis B virus (HBV)	HBV	Intravenous	Mouse
	Dyslipidaemia	ApoB	Intravenous	Monkey
	Ebola (Zaire)	Polymerase L	Intravenous	Guinea pig
Cationic polymers				
Cyclodextrin	Ewing's sarcoma tumour xenograft	*EWS–FLI1*	Intravenous	Mouse
	Healthy monkey model	RRM2	Intravenous	Monkey
Dynamic PolyConjugate	Dyslipidaemia	ApoB/PPARα	Intravenous	Mouse
Poly-ethyleneimine	Glioblastoma xenograft	PTN	Intratumoral	Mouse
	Formalin-induced pain	NMDAR2B	Intrathecal	Rat
	Cervical tumour xenograft	HPV E6/E7	Intratumoral	Mouse
	Ovarian tumour xenograft	HER2	Intraperitoneal	Mouse
Small interfering RNA (siRNA) conjugates				
Cholesterol	Dyslipidaemia	ApoB	Intravenous	Mouse
	Huntington's disease	Huntingtin gene	Intrastriatal	Mouse
Fatty acids/ bile salts	Dyslipidaemia	ApoB	Intravenous	Mouse, hamster

Cholesterol-conjugated siRNAs have shown to improve RNAi efficacy significantly both in cultured cells and *in vivo* (*46, 76, 77*). SiRNA conjugated to a single wall carbon nanotube efficiently delivered siRNA in cultured HeLa cells and lead to potent silencing of the lamin A/C gene (*78*).

To enhance the therapeutic potential of siRNA and reduce toxicity, targeted delivery is desired and required. Targeted delivery of siRNAs has been accomplished by anchoring an appropriate ligand (recognizable and taken up by a particular type of cell) to the delivery vehicle. Transferrin-anchored polycationic nanoparticles can selectively deliver siRNA into targeted cells by virtue of recognition of transferrin by specific receptors on the target cell surface (*79*). Folic acid (*80, 81*) or a specific carbohydrate,(*66, 82*) when anchored to PEG oligomers, can direct the delivery of siRNAs to selective target or diseased cells, because many cells overexpress folate or carbohydrate receptors under abnormal conditions. PEG-decorated liposomes (SNALPs) can selectively deliver siRNAs in the livers of non-human primates; in one case the RNAi efficacy is >90% and one dose lasts for 11 days, indicating prolonged half-life of the siRNA in that formulation (*47*).

Conjugation of cell-penetrating peptides with siRNAs has also helped reduce dosage and improve RNAi efficacy (*83*). SiRNAs, when conjugated to liposomes, antibodies or neuropeptides can overcome the blood-brain barrier and can be delivered into the brain (*3, 84*). Antibody (targeting HIV 1 envelope protein gp160)-conjugated protamines can bind siRNAs (targeting HIV gene gag) electrostatically, and deliver siRNAs inside an HIV-infected murine model (*42*). Aptamers are gradually gaining popularity as delivery vehicles for the siRNA (*85, 86*). Aptamers are *in vitro*-evolved

synthetically prepared nucleic acid oligomers that can bind selectively and tightly to specific ligands; therefore aptamers can help the intracellular delivery of other biomolecules through selective aptamers-ligand interactions, provided an aptamer is available for the specific ligand (receptor or a part of the receptor) on the cell-surface. Prostate-specific membrane antigen-binding aptamers when conjugated to siRNAs targeting pro-survival genes in prostate cancer cells have been found to deliver the RNAi agent efficiently inside cells (*43, 87*).

SiRNA therapeutics – pros and cons

SiRNA enjoys certain distinctive advantages over small molecule and protein based drugs. Small molecules have the limitation of being useful to "druggable" targets and the application of protein-based therapeutics is restricted to extracellular targets only; contrarily, RNAi can be extended to any biological target, including so called "non-druggable" targets. Like protein-based antibodies and unlike small molecules, siRNAs are very selective and potent. It is much easier to synthesize short RNA oligomers than synthesizing complex organic molecules of biological significance; siRNA lead optimization is also much faster compared to protein-based drugs.

In spite of all these advantages of siRNA over other therapeutics, the therapeutic potential of RNAi has not been fully exercised yet because of numerous problems associated, such as ineffective delivery, poor nuclease-stability and numerous undesired side effects. SiRNA molecules, unlike small molecules, have high negative charges in its backbone and hence cannot cross the cellular membrane without the aid of delivery vehicles. Either covalent conjugation with other groups or noncovalent assembly with other materials is essential for their intracellular delivery.

Optimal thermal and nuclease stability is essential for the therapeutic efficiency of siRNAs. Double stranded RNAs are much more stable compared to the single stranded ones toward nucleases. Generally, siRNAs are designed so that the duplex is of intermediate thermal stability – if the stability is very high, the RNAi machinery might not be able to unwind the siRNA for selective mRNA knock down; again, if the duplex is unstable (i.e., the melting temperature is low), then the siRNA will be an ineffective one since higher percentage of siRNA will exist as single strands which will be rapidly degraded by serum ribonucleases. Several chemical modifications have been reported to improve the thermal and the nuclease stability of siRNAs and these modifications do not affect the RNAi efficacy too much.

Native siRNAs can have off-target effects and instigate immunostimulation; these lower the specificity of siRNA action and induce toxicity inside cells. Several types of off-target effects can arise with siRNA-based therapeutics. Perfect complementarity of various regions of multiple mRNAs with the 'seed' region of an siRNA is a possible source of undesired side effect. To overcome this problem, several siRNAs are chosen as potential candidates and the one with optimal silencing efficacy and minimal ability to instigate non-target gene repression is chosen as the most promising compound. Judicious chemical modifications are another alternative to achieve RNAi selectivity and avoid undesired repression of non-target gene expression.

Chemical modifications of the siRNA

Analogous to the well-known antisense technology, chemical modifications of siRNA have promise in improving siRNA properties such as enhanced duplex stability, improved nuclease resistance, higher potency, higher specificity etc. Also, chemically

modified siRNAs have facilitated cellular uptake, enhanced half-life, improved biodistribution and better pharmacokinetics. Naked siRNAs are rapidly degraded and excreted through the urinary system. Therefore, suitable chemical modifications are required for the translation of siRNA into potent therapeutics; in fact a large proportion of the siRNAs currently in clinical trial are chemically modified (*6*). SiRNA modifications can be classified into several categories: ribose modifications, backbone modifications, base modifications and terminal modifications including conjugation to other biologically significant molecules.

Ribose modifications. Ribose modifications are the most explored chemical modifications of the siRNA; from subtle to extensive modification of the sugar moiety have been reported (Figure 1.5). Modifications of the ribose moiety (*88-105*) have improved siRNA stability (*88, 96*), ribonuclease resistance (*88, 90, 93, 95, 99*), potency (*90, 95, 104*), and specificity (*88, 106*). 2′-OH of the sugar moiety has been modified more frequently than any other positions. 2′-deoxy (*94*), 2′-F (*90*) or 2′-N_3 (*97*) substitutions did not significantly affect RNAi efficacy. 2′-Deoxy modifications lower the T_m of the siRNAs slightly, but are tolerated at the terminal of each strands and at the overhangs; however substitution of one of the strands with 2′-deoxy nucleosides (i.e., RNA:DNA hybrid) completely abolishes the RNAi activity (*94, 107*). 2′-NH_2 substitutions maintains RNAi activity, but to a lower extent than the unmodified siRNAs (*94*). 2′-NH_2 modifications led to reduction in RNAi efficacy when placed in the passenger strand and drastic reduction in efficacy when placed in the guide strand (*94*).

In earlier studies, 2′-OMe substitutions (*89*) have been reported to drastically reduce RNAi effects. Fully 2′-OMe-substituted nucleoside containing siRNAs were not

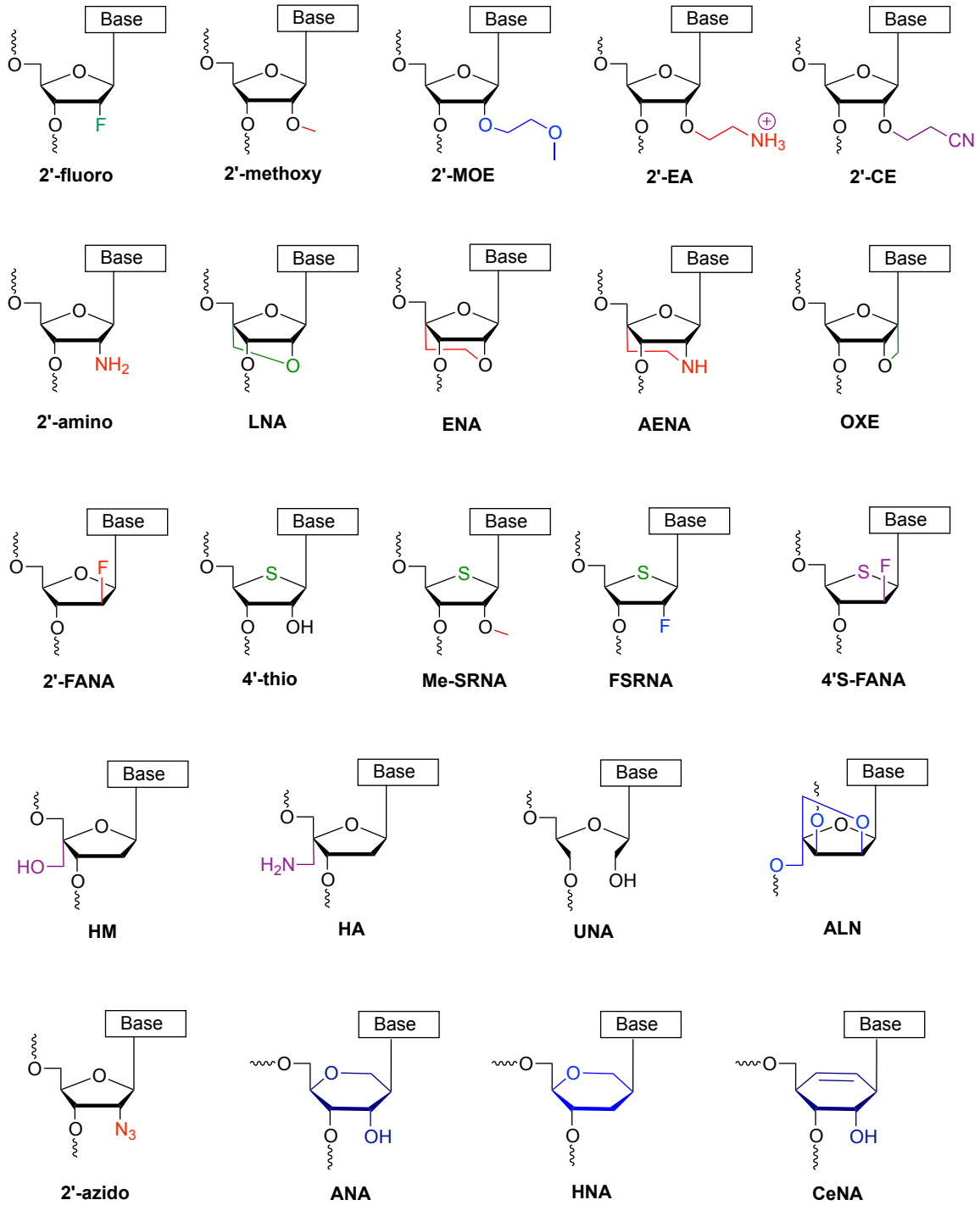

Figure 1.5. SiRNA ribose modifications.

functional, however two to four modifications in either strand were well tolerated. When every other nucleoside was modified with 2′-OMe, siRNAs were significantly resistant to serum nucleases (*108*). In another study, alternate placement of 2′-F and 2′-OMe modifications both in the guide and the passenger strands increased therapeutic efficacy of the siRNA (*90*). 2′-MOE (methoxyethyl) modifications were tolerated in the guide strand and alternate placement of 2′-MOE and 2′-OH or several 2′-MOE at the termini improved the RNAi activity (*109*). In general, the activity of 2′-sugar modifications in siRNAs depends on their size and position in the duplex; small modifications such as 2′-F are generally well tolerated in the guide strand regardless of their position (*92, 96*). Larger 2′-OMe modifications are well tolerated at the 3′-end of the guide strands and at any positions in the passenger strands. Bulky 2′-MOE modifications are better placed in the passenger strands, whereas their inclusion in the guide strand generally decreases RNAi efficacy (*92*). 2′-F (*88*) and locked nucleic acid (LNA) (*88*) (containing a methylene bridge between the 2′ and the 4′ positions of the ribose) modifications are now frequently used to enhance nuclease resistance, thereby increasing the potency and decreasing the dosage of siRNA. 2′-F, LNA and ENA (ethylene-bridge nucleic acid) are known to restrict the sugar conformation and thereby enhance the specificity of complementary strand recognition and the strength of target binding. These two modifications, along with the 2′-F modification can increase the melting temperature (T_m) of siRNAs significantly (*88*). SiRNAs containing LNAs at the 5′-end 3′-end or both in the same strand can exhibit RNAi activity (*88*); however, LNA substitution at the central region of the siRNA leads to complete loss of activity.

Substitution of the 4′-O with S has enhanced the T_m and the nuclease resistance of siRNA; 4′-S modified nucleosides are well tolerated at the termini of the both passenger and guide strands (*110, 111*). 4′-thio-2′-fluoroarabinonucleic acid (4′-S-2′-FANA) modifications, when placed into siRNAs, were able to mediate gene silencing through RNAi pathway; modifications at several sites afforded highly potent siRNAs (*98*). Alternate placement of 2′-FANA (2′-fluoroarabinonucleic acid) and 2′-FRNA (2′-fluororibonucleic acid) or LNA in an siRNA enhanced its potency (*104*). Recently the unlocked nucleic acid (UNA)-based sugar modifications were reported; these unrestrained groups can prevent sequence-dependent off-target effects at the expense of slight loss of efficacy (*106*). Few modifications of the sugar moiety have been reported in which the entire ribose group have been replaced by other groups as in the cyclohexenyl nucleic acid (CeNA), where one or two ribose moieties were replaced with cyclohexene moieties; resultant siRNAs retained their RNAi activity (*112*).

Backbone modifications. Phosphodiester backbone (*88, 89, 94*) modifications have improved several properties of the siRNA and made it more diverse as a therapeutic agent. Subtle phosphodiester backbone modifications, such as phosphorothioate (PS) and boranophosphate (BP) modifications, as well as complete replacement of the natural backbone with peptide or morpholino modifications have been reported (Figure 1.6). PS modifications are known to improve the ribonuclease resistance and pharmacokinetic profiles of the antisense oligonucleotides (*113*); these modifications are also known to bind with serum proteins and thereby enhance the half-life of the PS-modified antisense oligomers in the systemic circulation (*114*). Similar property enhancements might also be observed for siRNAs. However PS-modifications in the passenger strand or in the guide

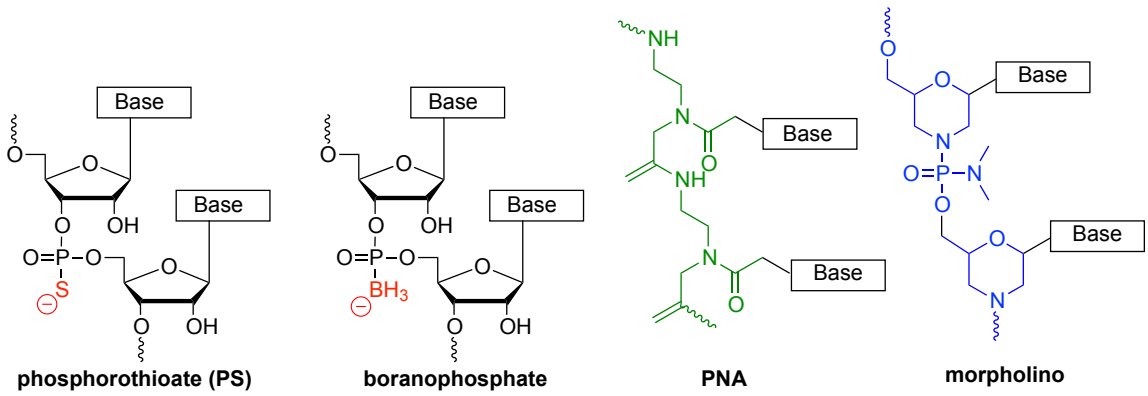

Figure 1.6. SiRNA backbone modifications.

strand lowers the T_m of the duplex (*88*) and reduces the RNAi efficacy (*89*) moderately. Fire and coworkers reported that phopsphorothioate substitution in both the guide and the passenger strands maintain RNAi effect in *Caenorhabditis elegans* (*94*). This reduction in RNAi potency may be associated with reduced binding interaction between the PS-modified siRNAs and the RISC complex (*100*). Extensive substitution of the backbone with PS group has been proven to be cytotoxic; however moderate numbers of PS modifications have been tolerated in siRNAs (*7*).

In the boranophosphate modifications, one of the nonbridging oxygens is replaced with a borane (BH_3) group. Enantiomerically pure boranophospate-substituted (BP) siRNAs are more nuclease resistant than racemic PS-substituted or unmodified siRNAs. BP modification slightly increases the T_m of the siRNA duplex and BP-modified siRNAs are more potent than PS-modified siRNAs. The BP-backbone is more lipophilic than the unmodified siRNAs and hence can have significant improvement in the pharmacokinetic and pharmacodynamic profiles of the BP-modified siRNAs compared to the unmodified ones. This modification is better tolerated in the passenger strand than in the guide strand and towards the termini of the oligonucleotides than the central region of the siRNA (*115, 116*).

In peptide nucleic acids (PNAs), the phosphodiester backbone is replaced by a peptide backbone. SiRNA-PNA chimeras at, or in place of the 3′ overhangs, have increased resistance to exonuclease digestion, siRNA half-life and RNAi efficacy (*117*). Morpholino (*118*) and triazolyl (*119*) modifications in those positions afforded similar results.

Terminal modifications and bioconjugates. Polymer- (*66, 82, 120*), lipid- (*121*), cholesterol- (*76, 122*), carbohydrate- (*66, 82*), peptide- (*123*) and small molecule- (*122*) based terminal modifications and delivery systems of siRNAs exhibited effective RNAi; polymer- and cholesterol-based conjugates facilitated intracellular delivery of siRNA both *in vitro* and *in vivo* (*46, 124*) (Figures 1.7 and 1.8). For target specific delivery of the siRNA, suitable ligands (e.g., folic acid (*81*), mannose (*82*), hyaluronic acid etc.) are chemically attached at the termini of the strands. Small lipid-like molecules (lipidoids) when terminally attached to siRNAs can mediate RNAi in cultured cells and in animal models without the necessity of external delivery vehicles (*74, 77*).

Base modifications. SiRNA base modifications are relatively less explored compared to ribose modifications or terminal modifications because preservation of necessary H-bonding interactions and stable A-form duplex are crucial in effective RNAi (Figure 1.9) (*9, 89, 125-131*). Disruption of necessary H-bonding interactions (e.g. in N^3-Me-U) or steric occlusion of siRNA-RISC interactions (5-IU) can adversely affect the RNAi efficacy and specificity (*89*). For this reason, most of the successful base modifications have been introduced into either passenger strands or at the 3′-end of the guide strands (*125, 129, 131*). Base modifications have some additional advantages that cannot be matched by other chemical modifications; the importance of base pairing (*126*) at certain location in the siRNA can only be explored by suitable base modifications. Similarly, sterically demanding base modifications (e.g., triazolyl-containing click adduct) are tolerated at sites where even a small ribose modification (e.g., 2′-OMe) abolishes the RNAi activity completely (*132*).

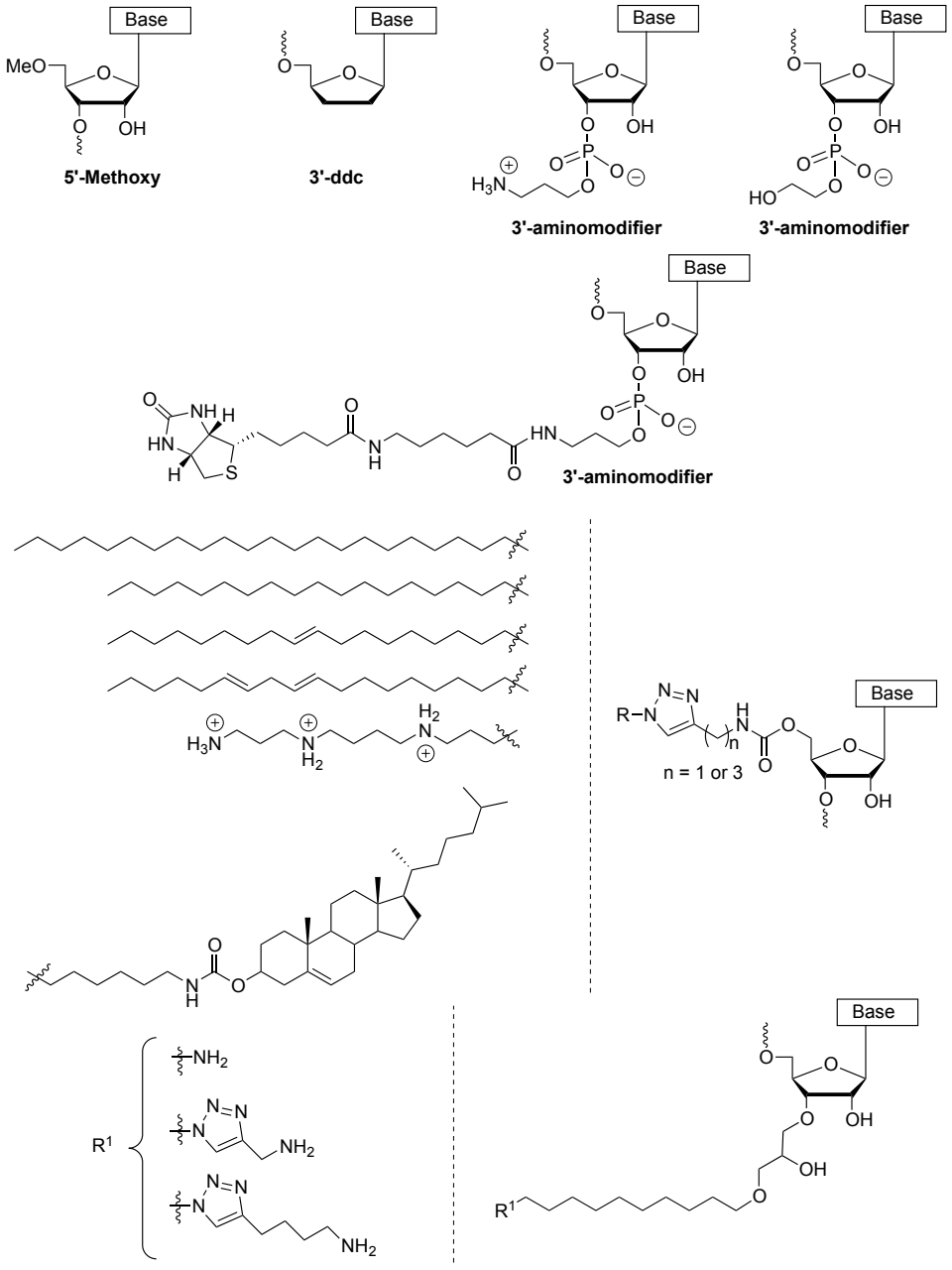

Figure 1.7. SiRNA terminal modifications.

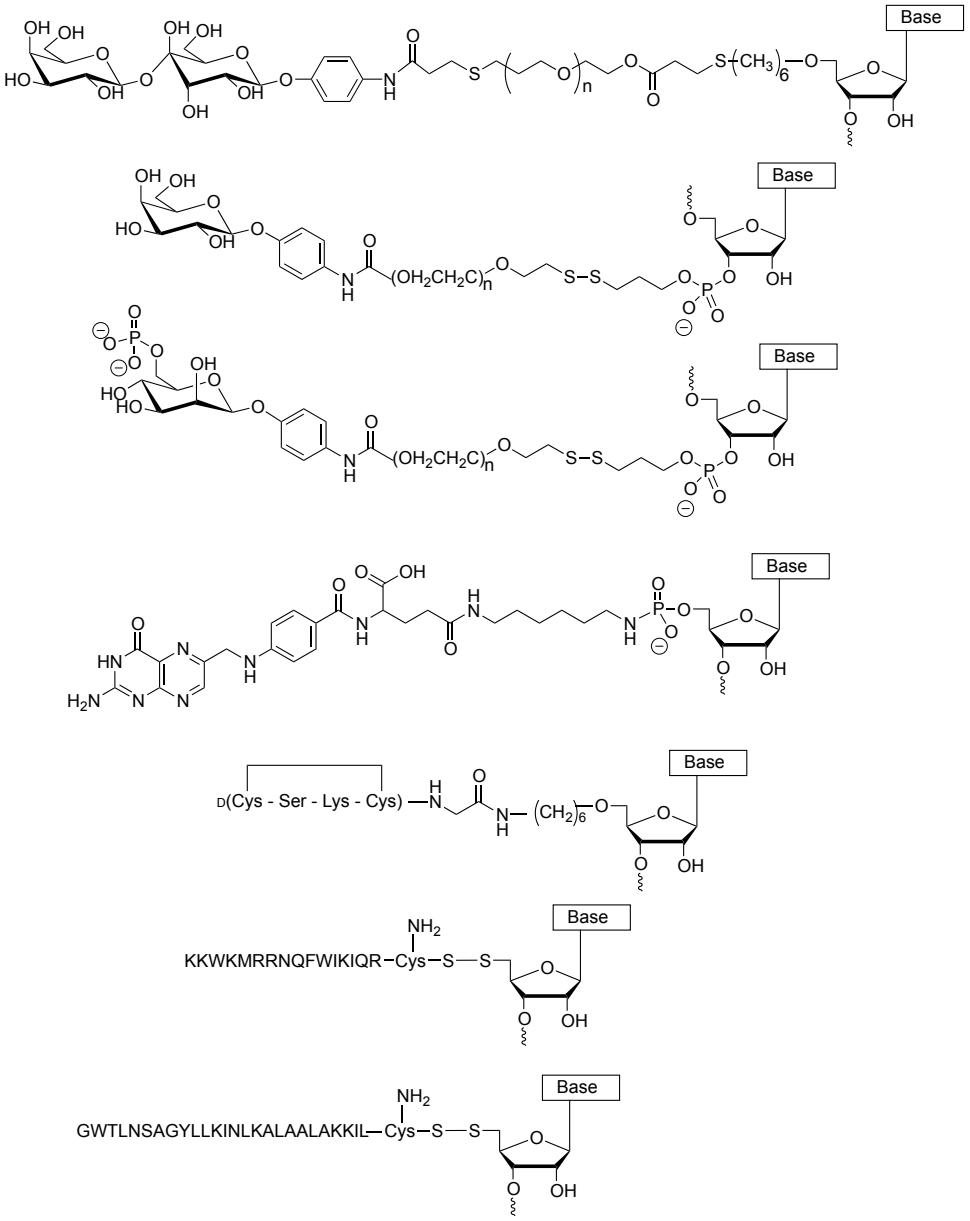

Figure 1.8. SiRNA bioconjugates.

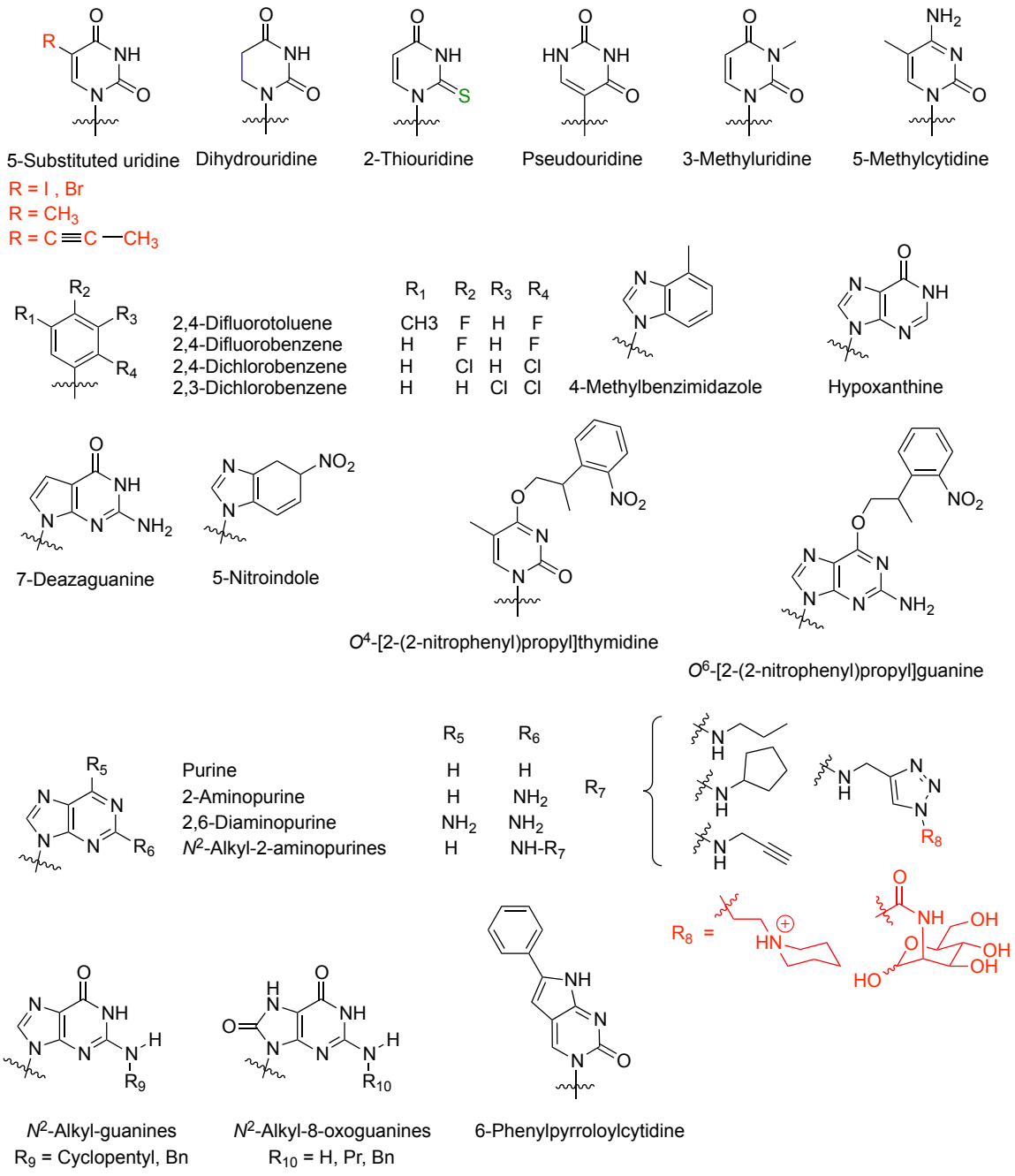

Figure 1.9. SiRNA base modifications.

Effect of H-bonding on siRNA activity was explored by utilizing nucleobase isosteres in place of natural bases (Figure 1.9) (*126, 127, 133*). These moieties are hydrophobic and incapable of forming hydrogen bonds with natural nucleobases. Two hydrophobic isosteres of U, 2,4-difluorobenzene and 2,4-dichlorobenzene, when placed in different regions of the guide strand, enhanced thermal stability and maintained moderate RNAi efficacy when placed in the terminal regions of the siRNA. Similarly, 2,4-difluorotoluene-containing siRNAs have enhanced nuclease stability and comparable RNAi activity (*127*). These modifications are well tolerated at position 7 of the guide strand, however when placed next to the mRNA cleavage site (position 10 or 11) RNAi efficacy is largely abolished (*126, 127*). 2,3-Dichlorobenzene and 4-methylbenz-imidazole, even though unable to form H-bonds with other nucleosides, prefer U as the complementary base in the mRNA; steric similarity of those modifications with A might be a plausible explanation for this observation (*133*).

Mayer and coworkers reported that introduction of thermally destabilizing groups such as, 2,4-difluorotoluene, hypoxanthine, 5-nitroindole, purine, and 2-aminopurine at various positions within the passenger strand improved RNAi efficacy mainly due to thermal destabilization of the duplex (*134*). RNA major groove-modulating alkyl groups (methyl and propargyl) when substituted in U or C enhanced the thermal and nuclease stability of the siRNAs; however their effect on RNAi was dependent on the position and size of the modification (*128*). 2-Thiouracil or pseudouracil at the 3′-terminal of the guide strand increases the RNAi efficacy when a single dihydrouracil is also placed at the 3′-end of the passenger strand (*135*); this enhancement in efficacy can be attributed to increased duplex asymmetry and facilitated strand unzipping from the 5′-end (*135*).

6-Phenylpyrrolocytosine, a highly fluorescent nucleoside analog, when placed in the passenger strand or towards the 3′-terminal of the guide strand, silenced target genes efficiently and helped visualize intracellular trafficking (*131*). Minor-groove-modulating guanine and 2-aminopurine modifications have been reported to reduce off-pathway protein interaction (*125, 129, 130*). Mikat and Heckel investigated the use of the photolabile 2-(2-nitrophenyl)propyl group as a temporary major groove modification of U or G in the guide and passenger strands of the siRNA; but those modifications were found to be fully effective only at a single site (*136*) .

Conclusion

The therapeutic potential of siRNA has not yet been exercised fully, mainly due to lack of appropriate delivery vehicles and numerous off-target effects. To address limitations of siRNA, chemical modifications of different segments of the siRNA are a potential solution. Although ribose modifications are most extensively studied, backbone and terminal modifications have also been explored. Terminal modifications of siRNA strands have served multiple purposes, e.g., intracellular delivery, enhancement of siRNA half-life, improving bio-distribution and pharmacokinetic profiles. Although not as common, successful base modifications have been reported both in the passenger and the guide strands. Base modifications provide valuable information regarding necessity of base pairing at certain regions of the siRNA, the scope of modification as applied to A, U, G, and C, the structure of the major and minor grooves during delivery and in the RISC and prevention of off-pathway protein binding.

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CHAPTER 2

8-ALKOXYADENOSINES AS POTENTIAL MODULATORS OF OFF-PATHWAY PROTEIN BINDING

Introduction

SiRNA-based RNAi pharmaceuticals are gradually becoming a principal alternative to small molecule-based drugs. Theoretically, any malfunctioning gene (and the related disease) can be targeted by this technique; even so called 'nondruggable' targets can be reached by siRNAs. RNAi is still a relatively new technology, and within a short time period significant advancement has been made to bring this technique from the research laboratory to the clinic.

However, the therapeutic potential of siRNA has not yet been maximized mainly due to delivery problems and undesired side effects, such as sequence-dependent off-target effects, sequence-specific and sequence-independent off-pathway protein binding leading to innate immune responses. Although not as pronounced as the long dsRNAs, siRNAs can induce an innate immune response. SiRNAs are recognized by the intracellular defense mechanism as a potential viral infection; and the genes related to the innate immune system (such as INF_{α}, 2′,5′-oligoadenylate synthase (OAS) and signal transducers/activators of transcription) are upregulated leading to immunostimulation (*1, 2*). These side effects can mask or even counter RNAi efficacy and specificity. Due to these off-target effects and off-pathway protein binding, the therapeutic dosage of the

siRNA must be increased significantly leading to dsRNA-related toxicity. Hence, prevention of these undesired side effects is essential.

Off-target gene silencing. Initially, siRNA was thought to be an extremely selective method of gene silencing; it was thought to be only functional if there was a perfect complementarity between the guide strand and the mRNA. Several studies have claimed that even a single mismatch with the mRNA completely abolished the RNAi activity (*4*).

However, it was gradually understood that siRNA could silence genes, partially or extensively, even when there is partial complementarity between the siRNA and the mRNA. Jackson et al. reported that this kind of miRNA-like effects could originate when any region of an mRNA is complementary to the seed region (bases at positions 2-8) of the guide siRNA (*5-7*). One probable solution to this 'off-target' effect is to select a different siRNA, the seed region of which is only complementary to the target mRNA. Sometimes this might not be trivial because many other parameters should also be satisfied to design a successful siRNA.

Another alternative is to chemically modify specific positions of the seed region to reduce the off-target effects. A 2′-OMe modification in the seed region significantly lowered the sequence-dependent off-target effects. This modification, when placed in position 2, is more effective than any other positions (*5*). Bramsen et al. reported several ribose modifications that are effective in preventing the sequence-dependent off-target effect; their study revealed that the UNA modification is the most effective modification out of ten different modifications (Figure 2.1) (*8*). The UNA modification weakens the siRNA-mRNA interaction moderately, without lowering the RNAi efficacy considerably.

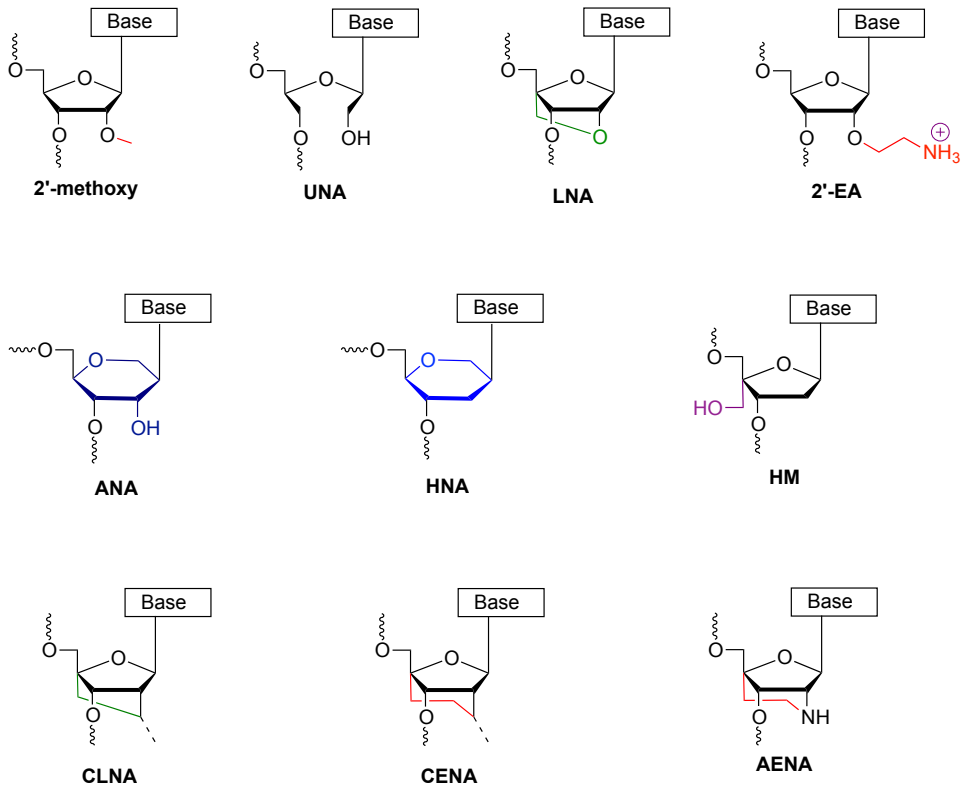


Figure 2.1. Ribose modifications to prevent off-target gene silencing (*8*).

Sequence-specific immunostimulation. Analogous to 5′-CpG-3′ containing DNA oligonucleotides (*9*), certain siRNA sequences are also immunostimulatory. GU-rich siRNA sequences produces Interferon-α (INF_α) which upregulates INF_α-associated genes in human peripheral blood mononuclear cells (PBMCs), plasmacytoid dendritic cells (PDCs) isolated from human peripheral blood, as well as in CD4+ 4 and CD8+ T cells in mice (*10, 11*). In these cases, immunostimulation occurs through interaction of Toll-like receptor 7 (TLR7) with certain sequence motifs in the guide or the passenger strand.

The mechanism of TLR-mediated immunostimulation due to siRNA binding is currently unknown. Several ribose modifications such as 2′-OMe, LNA, 2′-F-RNA, 2′-F-ANA, are known to prevent TLR-mediated immunostimulation (Figure 2.2) (*10, 12, 13*). To date, no base modification has been reported to address this off-pathway interaction. The TLR7-mediated innate immune response is sequence-dependent, so recognition of specific nucleoside motifs (such as GU-rich siRNAs) might be prevented by appropriate base modifications.

Sequence-independent off-pathway protein binding and immunostimulation. SiRNAs, irrespective of their sequences, can bind with proteins that are not directly involved in the RNAi pathway (*1, 14*). Among these proteins, an important class contains double-stranded RNA binding motif (dsRBM) containing domains, which can be used to bind duplex RNAs longer than 16 base pairs. Some important members of this group of proteins are RNA-dependent protein kinase (PKR) (Figure 2.3) and adenosine deaminases that modify RNA (ADARs), RNA helicase A, and Staufen1, an RNA trafficking protein. Duplex RNAs are also ligands of TLR3 and OAS and naturally can also interact with siRNA (*2, 15*). TLRs are a group of proteins that recognize molecular

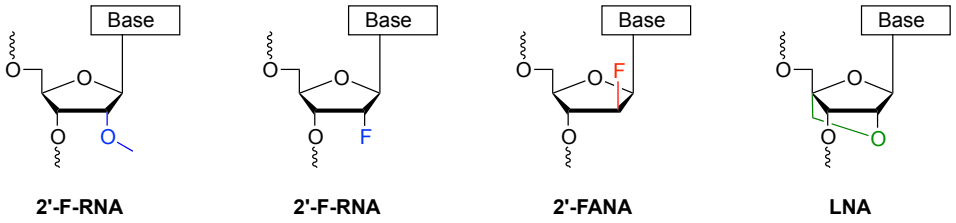


Figure 2.2. Ribose modifications to prevent sequence-specific immunostimulation (*13*).

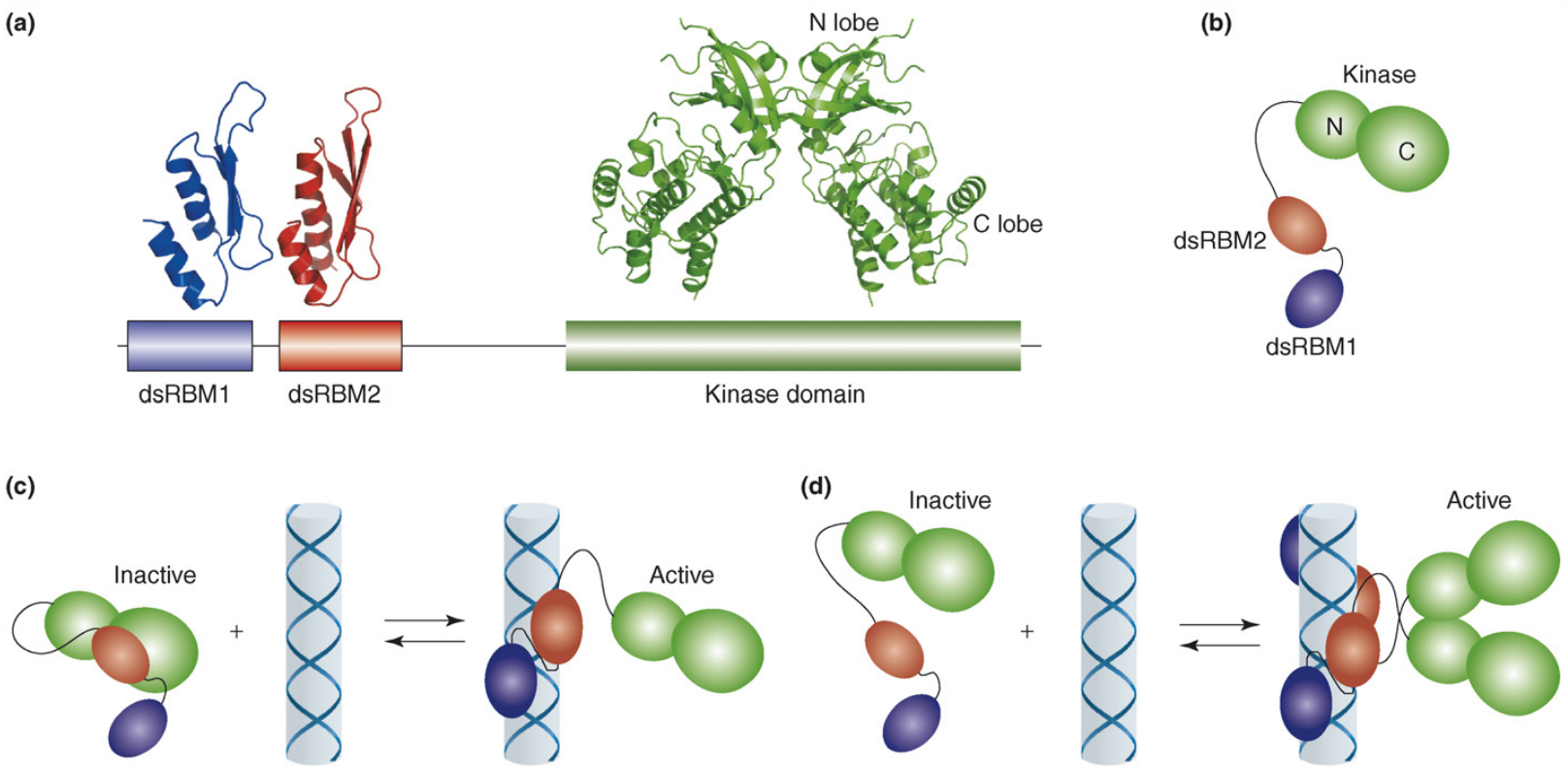


Figure 2.3. Structure and mechanism of activation of the RNA-dependent protein kinase (PKR). (a) and (b) structure of the PKR; (c) mechanism of activation of the monomeric PKR; (d) activation of PKR through dimerization (reproduced with permission from reference (*3*)).

scaffolds related to pathogenic infection and initiate immune response (*15*). OAS can be stimulated upon binding with siRNA and nonspecific Ribonuclease L is activated as a part of the innate immune response to viral infection (*16*).

PKR and ADAR1 have been reported to bind with siRNAs complicating RNAi experiments (*1, 14*). ADAR1 binds with the siRNA and reduces its effective concentration in cell, affecting RNAi efficacy significantly (*14*). PKR is a crucial component for controlling translation initiation and signal transduction; upon binding with siRNA, PKR sends signals analogous to viral infection and instigates interferon-mediated immunostimulatory response, which finally leads to cell apoptosis (Figure 2.3). Activation of PKR also induces global expression of PKR-associated genes in the human glioblastoma cell line (*1*).

Nucleobase modifications to prevent the siRNA-PKR interaction. Prevention of these sequence-independent off-pathway protein interactions is essential for the potency and specificity of therapeutic siRNA. The Beal laboratory initiated a systematic study on base modifications of the siRNA to address and alleviate siRNA-PKR interaction (*17, 18*). During PKR-binding studies with stem-loop IV of EBER1 (an RNA from Epstein-Barr virus), they noticed that substitution of some specific guanosines with N^2-benzylguanosine could greatly diminish PKR-EBER1 interaction. It was also realized that this strategy of 'steric occlusion of the minor groove' could be utilized to address siRNA off-target effects (Figure 2.4).

Their hypothesis was experimentally verified when modifications at several positions (6, 9, 11, 14) of the sense strand of caspase 2 siRNA successfully prevented PKR binding (Figure 2.4) (*18*). These N^2-benzylguanosine analogs maintained normal

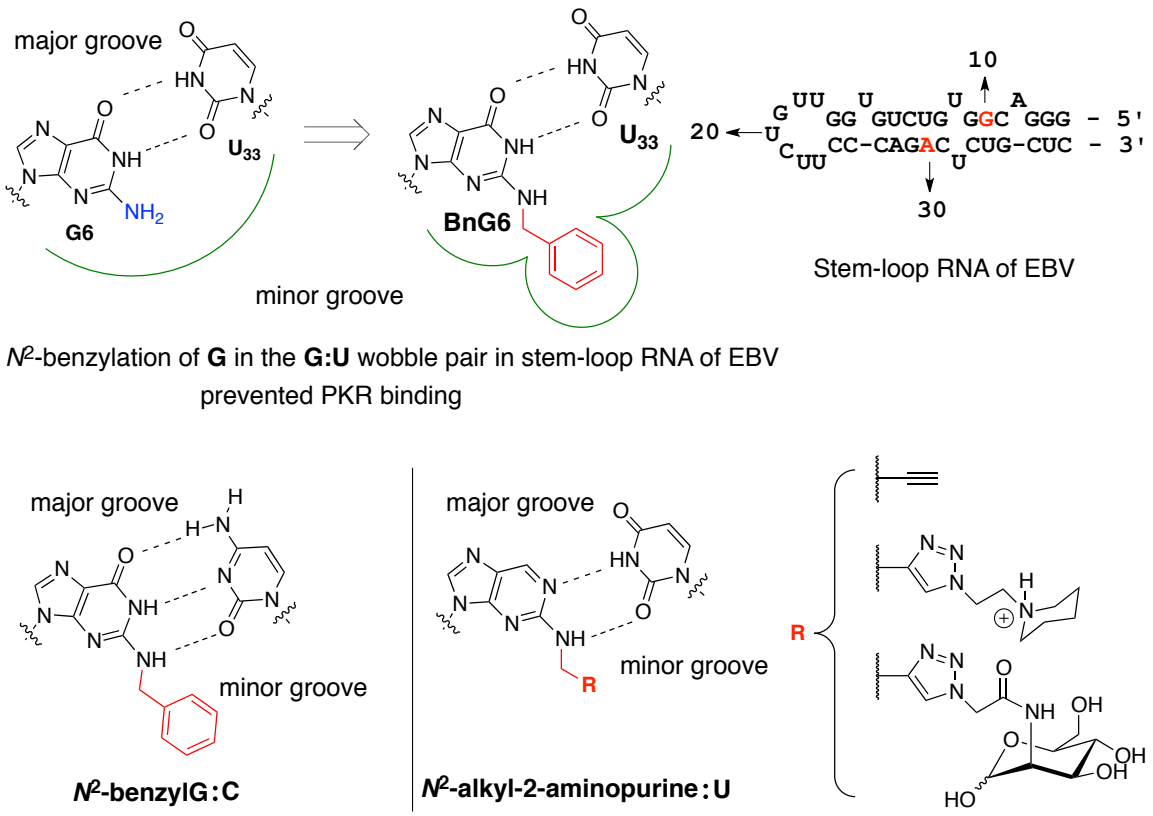


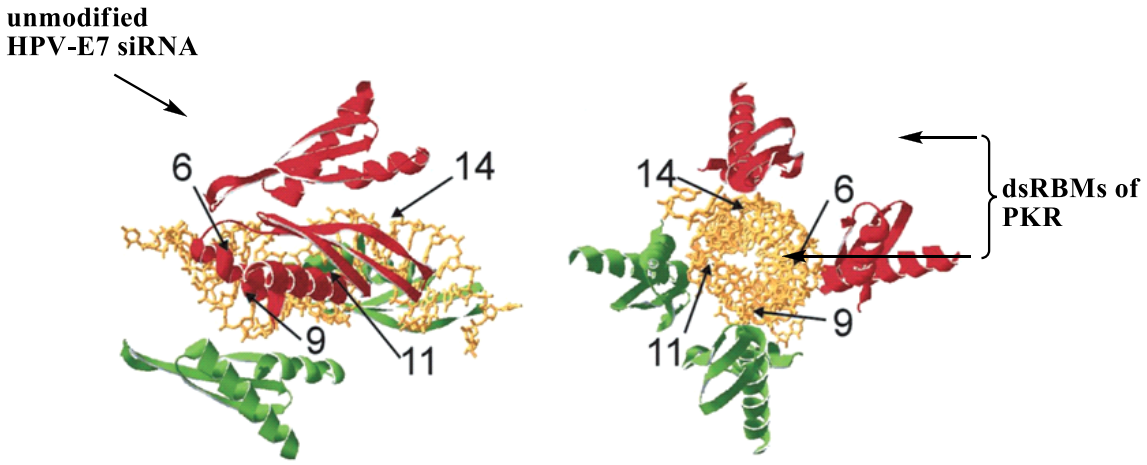
Figure 2.4. Minor groove purine modifications to prevent PKR binding (*18-20*).

hydrogen-bonded base pairing while projecting sterically demanding moieties in the minor groove; thereby preventing binding of dsRBM (of PKR) onto minor grooves of siRNA duplexes (Figure 2.5). Peacock et al. have shown (Figure 2.4) that pendent minor groove modifications of 2-aminopurines (*20*) in the passenger caspase 2 siRNA can also successfully prevent siRNA-PKR interaction, while maintaining RNAi efficacy.

Similar modifications of the minor or major groove in the guide strand might serve multiple purposes, such as preventing PKR or ADAR1 binding and elucidating novel mechanistic features of siRNA-RISC interactions while maintaining RNA interference activity. However, siRNA base modifications in the guide strand are more challenging; Some base modifications drastically reduced or completely abolished the RNAi efficacy when placed at crucial siRNA sites, such as in the seed region or at the cleavage site.

N^2-Alkylated 2′-deoxy-7,8-dihydro-8-oxoguanosine containing siRNAs could prevent siRNA-PKR interaction significantly by placing a 'switchable' alkyl group in the minor groove of an siRNA during delivery; in the RISC, the modified siRNAs can flip its steric bulk into the major groove, and maintain good siRNA efficacy (Figure 2.6) (*21*). However, oxidized purine lesions, such as 2′-deoxy-7,8-dihydro-8-oxoguanosine, are known to be immunostimulatory themselves. Therefore exploring other purine modifications in the guide strand is worthwhile and essential. Here, we report a series of novel nucleobase modifications, 8-alkoxyAs and their potential application in the RNAi.

8-AlkoxyA phosphoramidites were synthesized and incorporated into the guide strand of a caspase 2 siRNA through solid phase oligonucleotide synthesis. Modified siRNAs were tested for their RNAi efficacy and ability to address off-target effects due to siRNA-PKR interactions.



BndG modification at 6, 9, 11 and 14 positions of the sense strand disrupts PKR interactions.

Figure 2.5. Sites of the siRNA passenger strand modifications to prevent PKR binding. (reproduced with permission from reference (*18*)).

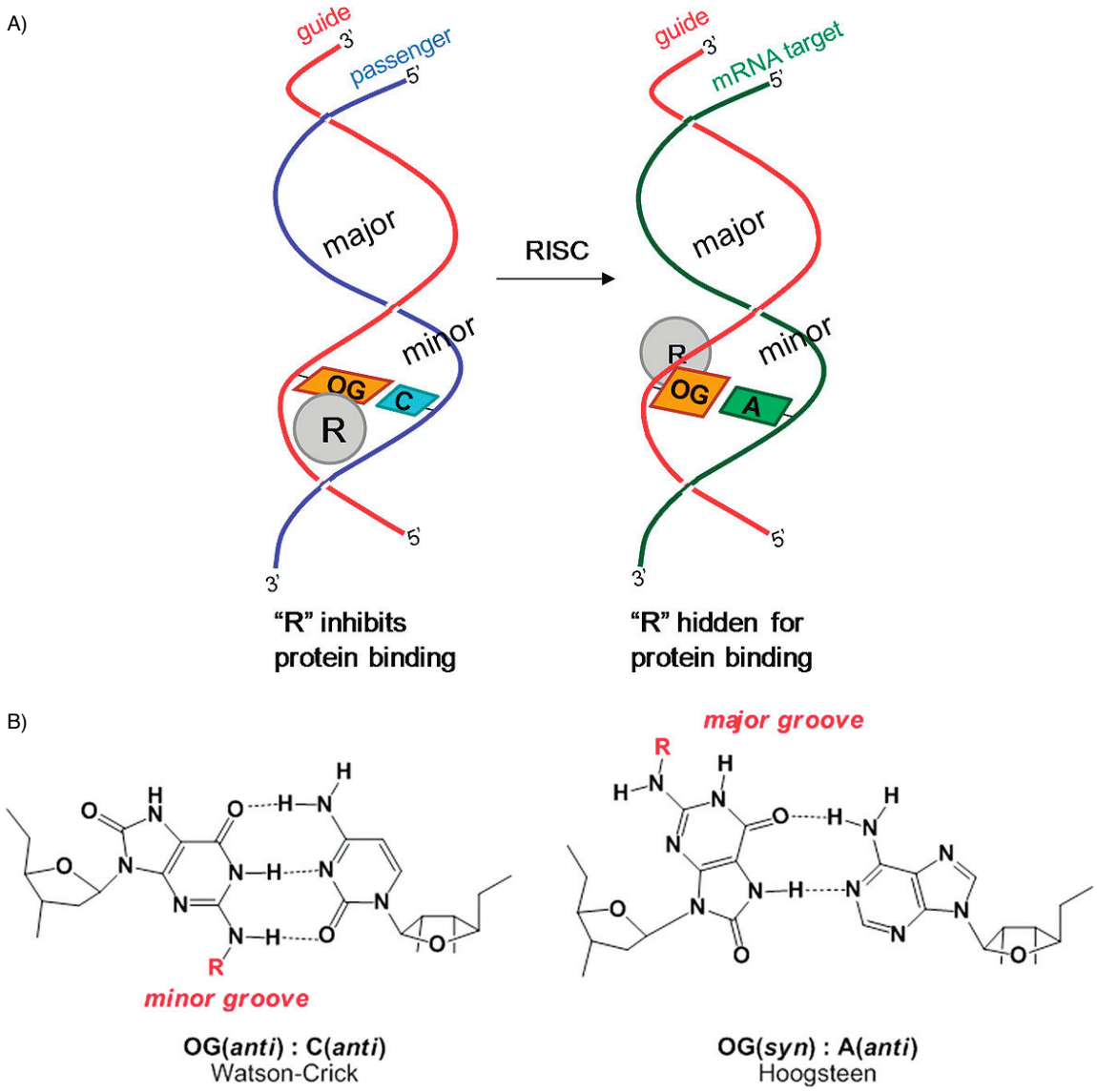
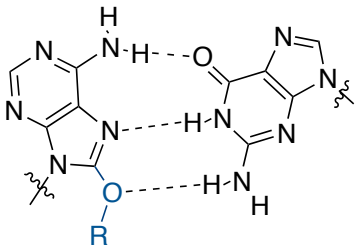


Figure 2.6. 'Switchable' N^2-alkylated 2′-deoxy-7,8-dihydro-8-oxoguanosines in duplex RNA to prevent PKR binding: (A) cartoon (B) base-pairs. (adapted and reproduced with permission from reference (*21*).

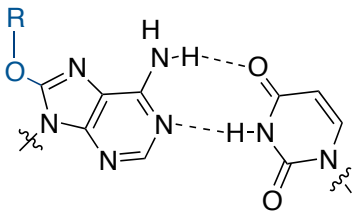
8-Substituted adenosines and guanosines have been shown to exist in an equilibrium mixture of *syn/anti* conformations. Accordingly, 8-alkoxyadenosines are postulated to have a tendency to flip between *anti* and *syn* conformations, depending on the base-pairing partner. In the natural *anti* conformation, 8-alkoxyA will base pair with U, whereas, in the *syn* conformation, the Hoogsteen face of the nucleoside will be exposed for base pairing and it will complement best with *anti* G (Figure 2.7). We propose that during delivery of the siRNA, 8-alkoxyA in the guide strand (opposite to G in the passenger strand) would project its steric blockade into the minor groove of siRNA, thereby preventing intracellular protein binding onto the RNA. When the siRNA is recruited into the RISC assembly, the 8-alkoxyA in the guide siRNA would encounter U in the mRNA and would flip its steric bulk into the major groove, thereby allowing necessary guide strand-mRNA-RISC assembly to form (Figure 2.8).

Alkyl groups were chosen based on their size and shape: propargyl, phenethyl and cyclohexylethyl. The rationale behind the choices is that: smaller groups (propargyl) might exhibit higher duplex stabilities and better mRNA knock down efficiencies whereas larger group (cyclohexylethyl) might prevent immunostimulation to a greater extent, and a medium-sized group (such as phenethyl) might serve both the purposes equally efficiently.

Synthesis of the 8-alkoxyadenosine phosphoramidites. 8-Alkoxyadenosine phosphoramidites were synthesized in multiple steps from a ribose-protected 8-bromoadenosine derivative. Here it is outlined briefly, and the detailed step- by-step synthesis can be found in the Experimental section below. Adenosine was first brominated at C8 following a published procedure and the hydroxyl groups of the ribose



8-alkoxyA$_{syn}$: G$_{anti}$



8-alkoxyA$_{anti}$: U$_{anti}$

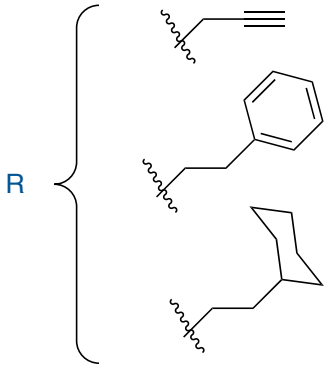


Figure 2.7. Proposed base-pairing of the 8-alkoxyadenosines.

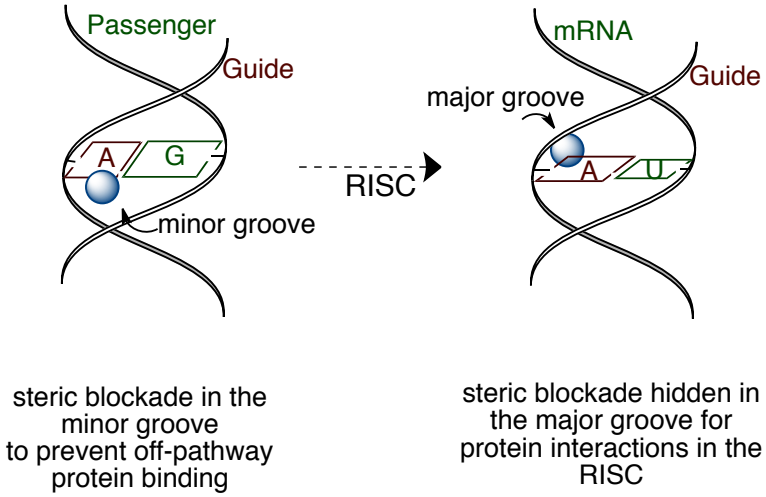


Figure 2.8. Proposed base 'switch' cartoon to prevent off-pathway protein interaction while maintaining RNAi efficacy.

moiety were protected by silylating agents. 5′-OH and 3′-OH groups were protected using a bidentate silylating agent and the remaining 2′-OH was protected using a TBDMS protecting group. Next, the bromine was displaced with an alkoxy groups that was generated in situ by adding dropwise *n*-BuLi into the corresponding alcohol in THF. The exocyclic amine of adenosine was protected using a benzoyl group. Then, the 5′-OH and 3′-OH were deprotected using pyridine-HF reagent, leaving the 2′-O-protection intact. For application in the automated solid phase oligonucleotide synthesis, the 5′-OH was protected with a DMT group, and the 3′-OH was coupled to a phosphoramidite group (Figure 2.9).

Experimental

All the chemicals were obtained commercially, unless otherwise stated, and used without further purification. Freshly distilled solvents were used where anhydrous solvents were required. THF was distilled from Na metal and benzophenone. For TLC, Merck silica gel 60 F254 pre-coated plates were used. Glassware for all the reactions was dried overnight in the oven at 150 °C and cooled in desiccators. Silica gel 60 (230-400 mesh) was used for flash chromatographic purpose. Column fractions were carried out as mentioned in the separation of individual compounds. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded at 300, 75, and 121 MHz, respectively. Chemical shift values are reported in parts per million (ppm) using the solvent peak as the reference. In interpreting the ^{1}H NMR peak multiplicities, s, d, dd, t, q, m, and brs abbreviations were used for singlet, doublet, doublet of doublets, triplet, quartet, multiplet, and broad singlet, respectively.

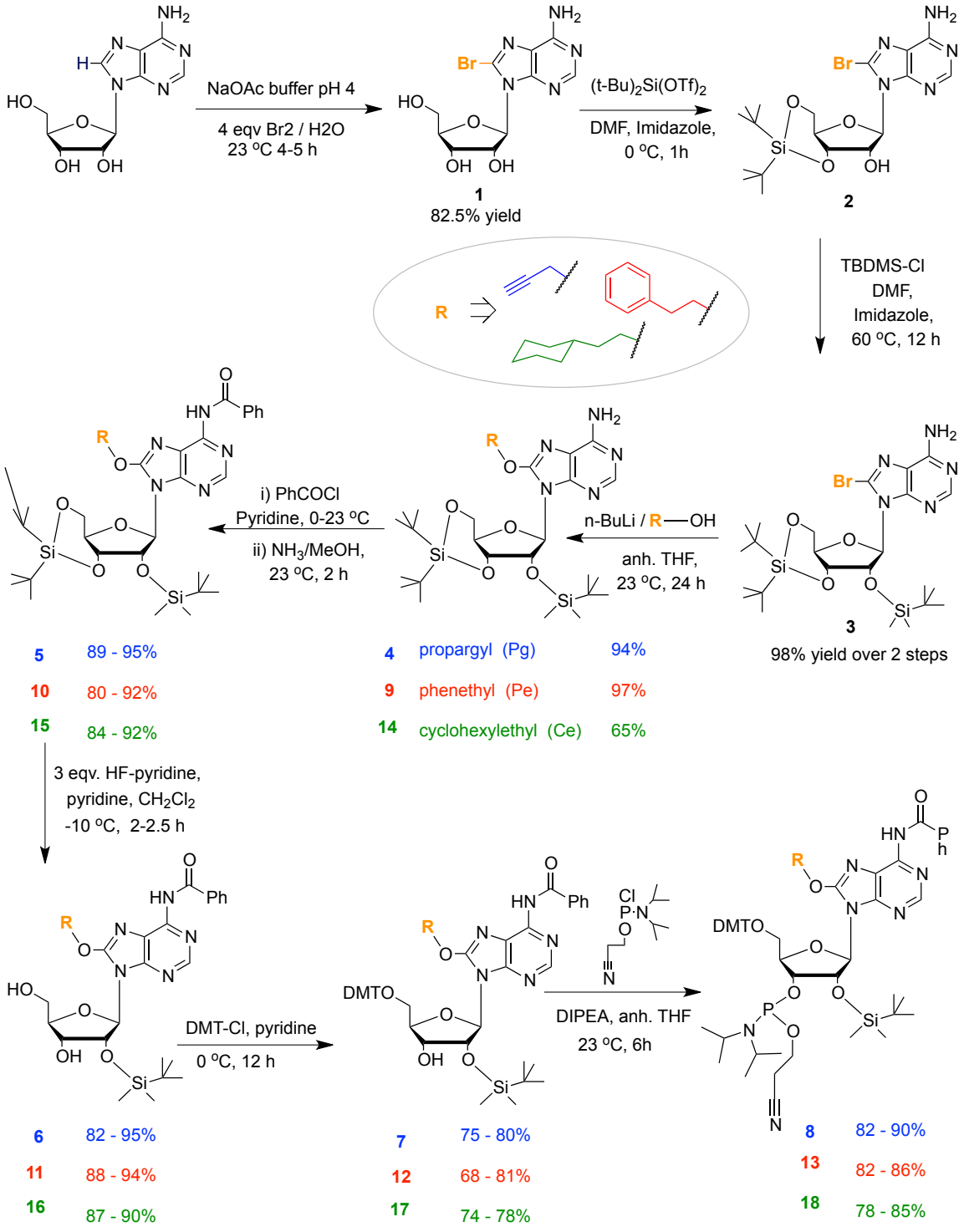


Figure 2.9. Synthesis of the 8-alkoxyA phosphoramidites.

8-Bromoadenosine (1). 8-Bromoadenosine was synthesized following a literature procedure (*22*). Product yield was 82.5%: melting point >200 °C; ^{1}H NMR (300 MHz, d_6-DMSO) δ 8.12 (s, 1H, 2-H), 7.56 (s, 2 H, 6-NH2), 5.80-5.84 (d, 1H, 1′-H), 5.42-5.52 (m, 2H, 2′- and 3′-OH), 5.20-5.25 (d, 1H, 5′-OH), 5.03-5.12 (q, 1H, 2′-H), 4.17-4.22 (brs, 1H, 3′-H), 3.94-4.00 (m, 1H, 4′-H), 3.62-3.72 (m, 1H, 5′-H), 3.46-3.56 (m, 1H, 5′-H). ^{13}C NMR (75 MHz, d_6-DMSO): δ 155.2, 152.4, 149.8, 127.2, 119.6, 90.4, 86.7, 71.1, 70.9. HRMS: calcd for $C_{10}H_{12}N_5O_4Na^{79}Br$ [MNa^+] 367.9970, obsd 367.9973.

5′,3′-*O*-Bis(-*t*-butylsilyl)-2′-*O*-(*t*-butyldimethylsilyl)-8-bromoadenosine(3). Both the 3′-OH and 5′-OH were protected by a designer protecting group introduced by Trost and coworkers (*23*). In an oven-dried flask 1 mmol (346 mg) of **1** was suspended in 5 mL anhydrous DMF. The suspension was cooled down to 0 °C and 1.1 equivalent (400 μL) di-*t*-butylsilyl ditriflate was added drop wise under stirring condition, at the same temperature. The reaction was carried out under N_2 atmosphere for 30 min and after that time period no starting material was detected, when the reaction mixture was analyzed using TLC. After synthesis of the intermediate **2**, the reaction was quenched immediately with 5 equivalents (344 mg) of imidazole at 0 °C. The reaction was stirred at the same temperature for 5 additional min, and then the system was allowed to equilibrate to room temperature. Then, 1.2 equivalents (181 mg) of *t*-butyldimethylsilyl chloride were added and a reflux condenser was connected to the reaction flask. The temperature was elevated to 60 °C, and the reaction was run overnight under nitrogen. The suspension was cooled down to room temperature, water was added and the precipitate was collected by suction filtration. The supernatant was discarded, and then the white precipitate was rewashed with cold methanol (4 °C) to obtain pure compound **3**. The methanol layer was

evaporated under reduced pressure and a simple column separation (5:1 hexane:ethyl acetate) could separate the remaining product. Overall yield of the product was 590 mg (98%): silica gel TLC R_f 0.44 (3:2 hexane:ethyl acetate); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.25 (s, 1H, 2-H), 6.55 (s, 2 H, 6-NH2), 5.89-5.92 (d, 1H, 1′-H), 5.15-5.24 (q, 1H, 2′-H), 4.86-4.92 (d, 1H, 3′-H), 4.33-4.42 (dd, 1H, 4′-H), 3.95-4.14 (m, 2H, 5′-H), 1.16 (s, 9H, $(CH_3)_3$), 1.03 (s, 9H, $(CH_3)_3$), 0.88 (s, 9H, $(CH_3)_3$), 0.11 (s, 3H, CH_3), 0.07 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.9, 153.3, 150.7, 127.6, 120.5, 94.2, 75.2, 74.6, 67.7, 27.8, 27.3, 26, 23, 20.5, 18.6, -4.2, -5.0. HRMS: calcd for $C_{24}H_{42}N_5O_4NaSi_2{}^{79}Br$ [MNa^+] 622.1856, obsd 622.1850.

5′,3′-*O*-Bis(*t*-butylsilyl)-2′-*O*-(*t*-butyldimethylsilyl)-8-propargyloxyadenosine (4). Propargyloxy anion was generated *in situ* by allowing n-BuLi to react with excess anhydrous propargyl alcohol. 0.83 mmol (500 mg) of compound **4** was dissolved in 2 mL anhydrous THF in a round bottom flask under argon gas. Anhydrous propargyl alcohol (1 mL) was added to 4 mL freshly distilled THF in a reaction flask fitted with a balloon; the solution was kept under nitrogen atmosphere and cooled down to -40 °C. Then, 10 mmol *n*-BuLi (3.5 ml of 2.5M hexane solution) was gradually added to the reaction mixture; the by-product butane was collected in a balloon. The reaction was completed instantly. This *in situ* generated lithium propargyloxide was transferred to the flask containing **4** through an oven-dried syringe. The reaction was allowed to proceed for 20 h at room temperature. TLC monitoring indicated the absence of any **4** after that time period. The solution was neutralized by dilute acetic acid. Excess solvent was removed under reduced pressure and the solid residue was then partitioned between water and ethyl acetate. The ethyl acetate layer was collected and dried by using anhydrous Na_2SO_4. Flash chromatography was

used to elute (2:3 hexane:ethyl acetate) the desired compound, **4**. Yield of 4 was 94%; silica gel TLC R_f 0.24 (2:3 hexane:ethyl acetate);^{1}H NMR (300 MHz, $CDCl_3$): δ 8.21 (s, 1H, 2-H), 5.93 (d, 1H, 1′-H), 5.61(s, 2H, NH_2), 5.05-5.20 (m, 2H, CH_2) 4.91-4.97 (q, 1H, 2′-H), 4.66-4.71 (d, 1H, 3′-H), 4.34-4.45 (m, 1H, 4′-H), 4.02-4.12 (m, 2H, 5′-H), 2.61-2.65 (t, 1H, CH), 1.00-1.20 (d, 18H, $(CH_3)_3$), 0.89 (s, 9H, $(CH_3)_3$), 0.11 (d, 6H, $2CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 153.7, 153.2, 151.8, 149.8, 116.0, 90.5, 75.3, 74.8, 74.7, 67.9, 58.0, 27.8, 27.2, 26.1, 22.9, 20.7, 18.8, -4.2, -4.9. HRMS: calcd for $C_{27}H_{46}N_5O_5Si_2$ [MH^+] 576.3038, obsd 576.3030.

5′,3′-*O*-Bis(*t*-butylsilyl)-2′-*O*-(*t*-butyldimethylsilyl)-N^6-benzoyl-8-propargyl-oxyadenosine (5). The N^6-amino group of **4** was protected using benzoyl chloride (BzCl). 0.77 mmol (435 mg) of **4** was dissolved in 2 mL anhydrous pyridine in a reaction flask and the solution was cooled down to -5 °C (by using salt-ice bath). Two equivalents of BzCl (1.54 mmol, 181μL) were added dropwise using a disposable syringe while stirring. The reaction mixture was allowed to warm to room temperature over a period of 1 h, and the reaction was continued for 4 h under argon. After that time, excess benzoyl chloride was quenched with 1 mL methanol. The reaction was allowed to stand for an h at 0 °C. Then, excess methanolic ammonia (1 mL 7N NH_3) was added and the solution was stirred for an additional hour at the same temperature. The crude product mixture was then dried down under reduced pressure to a solid residue, which was first partitioned between ether and saturated $NaHCO_3$ solution. The organic layer was then washed several times with water and, subsequently, dried with anhydrous Na_2SO_4. Product was then separated (3:1 hexane:ethyl acetate) by flash chromatography. Yield of the product **5** varied between 89% to 95%; silica gel TLC R_f 0.62 (2:3 hexane:ethyl

acetate); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.70-8.78 (bs, 1H, CONH-), 8.63 (s, 1H, 2-H), 7.95-8.02 (m, 2H, PhCO), 7.48-7.65 (m, 3H, PhCO), 5.91 (s, 1H, 1′-H), 5.13-5.29 (m, 2H, CH_2), 4.86-4.95 (q, 1H, 2′-H), 4.68-4.74 (d, 1H, 3′-H), 4.35-4.47 (m, 1H, 4′-H), 4.03-4.16 (m, 2H, 5′-H), 2.60-2.63 (t, 1H, CH), 1.13 (s, 9H, $(CH_3)_3$), 1.05 (s, 9H, $(CH_3)_3$), 0.90 (s, 9H, $(CH_3)_3$), 0.07-0.15, (d, 6H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.9, 154.9, 153.1, 151.3, 146.7, 134.2, 132.8, 129.1, 128.0, 120.5, 90.7, 75.5, 75.0, 67.8, 58.8, 27.8, 27.3, 26.1, 22.9, 20.6, 18.7, -4.2, -4.9. HRMS: calcd for $C_{34}H_{50}N_5O_6Si_2$ [MH^+] 680.3300, obsd 680.3297.

2′-*O*-*t*-Butyldimethylsilyl-N^6-benzoyl-8-propargyloxyadenosine(6). Compound **5** was selectively deprotected at 5′-OH and 3′-OH by using $(HF)_x$.py at subzero temperature. 0.74 mmol (505 mg) of **5** was added in CH_2Cl_2 and the solution was cooled down to -15 °C. 3.7 mmol (0.1 mL) of of $(HF)_x$•py was diluted with 600 μL pyridine at 0 °C. The latter solution was then gradually added to the former solution and the reaction temperature was maintained at -15 °C. The reaction was complete in 2-2.5 h. 50 ml CH_2Cl_2 was added to the product mixture. The solution was then washed with saturated aqueous $NaHCO_3$ solution. CH_2Cl_2 layer was then washed several times with water and dried with anhydrous Na_2SO_4. Pure product was obtained by flash chromatography (3:7 hexane:ethyl acetate). Yield of the desired product **6** was 82% to 95%; silica gel TLC R_f 0.13 (2:3 hexane:ethyl acetate);^{1}H NMR (300 MHz, $CDCl_3$): δ 8.99 (s, 1H, CONH-), 8.6(s, 1H, 2-H), 7.91-7.98 (m, 2H, PhCO), 7.42-7.58 (m, 3H, PhCO), 5.94-6.02 (d, 1H, 5′-OH), 5.89-5.93 (d, 1H, 1′-H), 5.12-5.16 (m, 2H, CH_2), 5.03-5.09 (m, 1H, 2′-H), 4.27-4.32 (m, 2H, 3′-H and 4′-H), 3.85-3.93 (bd, 1H, 5′-H), 3.64-3.74 (bt, 1H, 5′-H), 2.94 (s, 1H, 3′-OH), 2.60-2.64 (t, 1H, CH), 0.76 (s, 9H, $(CH_3)_3$), -0.18 (s, 3H, CH_3), -0.37 (s, 3H,

CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.7, 155.2, 150.9, 150.5, 147.4, 133.9, 132.7, 128.9, 127.8, 120.4, 87.9, 87.3, 76.3, 73.9, 72.7, 63.4, 58.4, 25.8, 17.8, -5.2, -5.3. HRMS: calcd for $C_{26}H_{34}N_5O_6Si$ [MH^+] 540.2278, obsd 540.2277.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*t*-butyldimethylsilyl)-N^6-benzoyl-8-proparg-yloxyadenosine (7). 0.204 mmol (110 mg) of compound **6** was dissolved in 2 mL anhydrous pyridine. The solution was cooled down to 0 °C under argon and 1.2 equivalent (85 mg) of DMT-Cl was added. The reaction mixture was stirred for 4 h at 0 °C and then for 6 h at room temperature. Then 1.2 equivalent of the DMT-Cl reagent was again added to the reaction mixture at 0 °C and the reaction was allowed to continue overnight. The reaction was quenched by addition of excess anhydrous methanol (0.5 mL). After another hour at room temperature, the solution was concentrated to dryness under reduced pressure. The crude solid was first fractioned between aqueous $NaHCO_3$ and ethyl acetate, and then the organic layer was washed several times with water. A short chromatography column (2:3 hexane:ethyl acetate) was necessary to purify the product. Yield of the product **7** varied from 75% to 80%; silica gel TLC R_f 0.47 (2:3 hexane : ethyl acetate); ^1H NMR (300 MHz, $CDCl_3$): δ 8.91 (s, 1H, CONH-), 8.5 (s, 1H, 2-H), 7.94-8.02 (m, 2H, PhCO), 7.14-7.6 (m, 12H, Ph), 6.74-6.81 (dd, 4H, Ph), 5.97-6.03 (d, 1H, 1′-H), 5.0-5.20 (dq, 2H, CH_2), 4.39-4.44 (t, 1H, 2′-H), 4.18-4.24 (q, 1H, 3′-H), 4.07-4.15 (q, 1H, 4′-H), 3.75 (s, 6H, OCH_3), 3.45-3.52 (m, 1H, 5′-H), 3.28-3.37 (dd, 1H, 5′-H), 2.78-2.84 (d, 1H, 3′-OH), 2.54-2.58 (t, 1H, CH), 0.83 (s, 9H, $(CH_3)_3$), -0.01 (s, 3H, CH_3), -0.17 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 165, 158.6, 155.7, 152.4, 151.2, 146.8, 145.1, 136.3, 136.1, 134.3, 132.8, 130-130.4 (d), 129.1, 128.4, 128, 127,

120, 113, 87.2, 86.5, 84, 72.8, 71.6, 63.8, 58.6, 55.4, 25.8, 18, 14.4, -4.6, -4.8. HRMS: calcd for $C_{47}H_{51}N_5O_8SiNa$ [MNa^+] 864.3405, obsd 864.3405.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-[(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphino]-2′-*O*-(*t*-butyldimethylsilyl)-N^6-benzoyl-8-propargyloxyadenosine (8). 8-Propargyloxyadenosine phosphoramidite was synthesized using the 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite reagent. 0.178 mmol (150 mg) of compound **7** (was dissolved in 5 mL of freshly distilled THF contained in an oven-dried flask. Five equivalent (0.9 mmol, 205 μL) DIPEA was added and the solution was stirred for 5 min at room temperature (23 °C), and then cooled down to 0 °C, under argon atmosphere. 81 μL (0.36 mmol, 2 equivalent) of phosphoramidite was added dropwise into the flask. Reaction was stirred at 0 °C for 30 min and then gradually warmed up to room temperature. After another hour under inert atmosphere, the solvent was evaporated by rotary evaporation and the desired product was separated by silica gel column chromatography (11:9 hexane:ethyl acetate). Yield of **8** varied from 82% to 90%; silica gel TLC R_f 0.47 (2:3 hexane:ethyl acetate); ^{1}H NMR (300 MHz, $CDCl_3$): 8.76-8.82 (d, 1H, CONH-), 8.44-8.52 (d, 1H, 2-H), 7.94-8.02 (m, 2H, PhCO), 7.14-7.62 (m, 12H, Ph), 6.74-6.82 (m, 4H, Ph), 5.95-6.02 (t, 1H, 1′-H), 5.29-5.40 (m, 1H, 2′-H), 5.02-5.4 (m, 2H, CH_2), 4.3-4.55 (m, 2H, 3′-H and 4′-H), 4.07-4.24 (q, 1H, 5′-H), 3.78-4.02 (m, 1H, 5′-H), 3.75 (s, 6H, OCH_3), 3.51-3.68 (m, 4H, CH_2CN, 2CHN), 2.64-2.70 (m,1H,P-O-CH_2), 2.50-2.56 (t, 1H, CH), 2.28-2.34 (m, 1H, P-O-CH_2), 1.14-1.3 (m, 12H, $4CH_3$), 0.70-0.80 (d, 9H, $(CH_3)_3$), (-0.08)-0.0 (d, 3H, CH_3),(-0.34)-(-0.20) (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.8, 158.6, 156, 155.9, 152.4, 152.3, 151.2, 146.8, 145.1, 144.9, 136.4-136.1(m), 134.4, 132.8, 130.4, 129.1, 128.5, 127, 120.1, 117.6, 117.9, 113.3, 86.4-87.0

(q), 84.0 (d), 72-73.8 (m), 63.4-63.8(d), 60.8, 59.0-59.4, 58.4, 57.8-58.4, 55.5, 43.5-43.8 (d), 43.0-43.4 (m), 25.9, 24.7-25.2 (m), 22.8, 23.4, 21.4, 20.7, 20.3, 18.2, 14.5, -4.3, -4.8. ^{31}P NMR (CD_2Cl_2): 149.7, 151.8. HRMS: calcd for $C_{56}H_{68}N_7O_9SiPNa$ [MNa^+] 1064.4483, obsd 1064.4493.

All phenylethoxyadenosine and cyclohexylethoxyadenosine derivatives were prepared following procedures analogous to those used for the propargyloxyadenosine derivatives.

5′,3′-*O*-Bis(*t*-butylsilyl)-2′-*O*-(*t*-butyldimethylsilyl)-8-phenylethoxyadenosine (9). Yield 97%; silica gel TLC R_f 0.32 (1:1 hexane:ethyl acetate); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.19 (s, 1H, 2-H), 7.23-7.36 (m, 5H, Ph), 5.85 (s, 1H, 1′-H), 5.52 (s, 2H, NH_2), 4.90-4.98 (q, 1H, 2′-H), 4.67-4.76 (m, 3H, 3′-H, OCH2), 4.34-4.41 (m, 1H, 4′-H), 3.95-4.10 (m, 2H, 5′-H), 3.12-3.2 (t, 2H, CH_2), 1.00-1.16 (d, 18H, $(CH_3)_3$), 0.87 (s, 9H, $(CH_3)_3$), 0.02-.10 (d, 6H, $2CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.2, 153.4, 151.5, 149.8, 116.0, 90.5, 75.5, 74.6, 71.1, 67.9, 35.2, 27.6, 27.2, 26.1, 22.9, 20.7, 18.6, -4.2, -4.9.

5′,3′-*O*-Bis(*t*-butylsilyl)-2′-*O*-(*t*-butyldimethylsilyl)-N^6-benzoyl-8-phenylethoxyadenosine (10). Yield 80-92%; silica gel TLC R_f 0.67 (2:3 hexane:ethyl acetate); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.80-8.88 (bs, 1H, CONH-), 8.59 (s, 1H, 2-H), 7.94-8.02 (m, 2H, PhCO), 7.46-7.62 (m, 3H, PhCO), 7.24-7.36 (m, 5H, Ph), 5.91 (s, 1H, 1′-H), 4.88-4.96 (dd, 1H, 2′-H), 4.76-4.84 (t, 2H, CH_2), 4.68-4.74 (d, 1H, 3′-H), 4.35-4.42 (q, 1H, 4′-H), 3.95-4.13 (m, 2H, 5′-H), 3.13-3.22 (t, 2H, CH_2), 1.2 (s, 9H, $(CH_3)_3$), 1.05 (s, 9H, $(CH_3)_3$), 0.89 (s, 9H, $(CH_3)_3$), 0.03-0.11 (d, 6H, $2CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 165, 156, 152, 151, 146.4, 137, 134.3, 132.8, 129, 128.0, 127 121, 90.6, 75.5, 74.5, 71.8,

67.8, 35.2, 27.8, 27.3, 26.1, 23, 20.7, 18.7, -4.2, -4.9. HRMS: calcd for $C_{39}H_{55}N_5O_6NaSi_2$ [MNa^+] 768.3589, obsd 768.3600.

2′-*O*-*t*-Butyldimethylsilyl-N^6-benzoyl-8-phenylethoxyadenosine (11). Yield 88-94%; silica gel TLC R_f 0.12 (2:3 hexane:ethyl acetate); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.88 (bs, 1H, CONH-), 8.62 (s, 1H, 2-H), 7.94-8.0 (m, 2H, PhCO), 7.44-7.62 (m, 3H, PhCO), 7.22-7.34 (m, 5H, Ph), 5.98-6.06 (d, 1H, 5′-OH), 5.87-5.92 (d, 1H, 1′-H), 5.07-5.14 (m, 1H, 2′-H), 4.62-4.82 (m, 2H, CH_2), 4.29-4.35 (d, 2H, 5′-H), 4.05-4.13 (q, 1H, 3′-H), 3.87-3.97 (d, 1H, 4′-H), 3.10-3.18 (t, 2H, CH_2), 2.90 (s, 1H, 3′-OH), 0.77 (s, 9H, $(CH_3)_3$), -0.16 (s, 3H, CH_3), -0.40 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.7, 156.2, 151, 150.5, 147.2, 136.9, 134.2, 133, 129, 127.2, 121, 87.4-88 (d), 72-74 (t), 35.4, 25.7, 17.8, -5.0, -5.3. HRMS: calcd for $C_{31}H_{39}N_5O_6NaSi$ [MNa^+] 628.2567, obsd 628.2575.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*t*-butyldimethylsilyl)-N^6-benzoyl-8-phenyl-ethoxyadenosine (12). Yield 68-81%; silica gel TLC R_f 0.6 (2:3 hexane:ethyl acetate); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.68 (bs, 1H, CONH-), 8.47 (s, 1H, 2-H), 7.94-8.02 (m, 2H, PhCO), 7.42-7.62 (m, 5H, Ph), 7.14-7.38 (m, 12H, Ph), 6.72-6.8 (dd, 4H, Ph), 5.87-5.92 (d, 1H, 1′-H), 5.26-5.32 (m, 1H, 2′-H), 4.66-4.80 (M, 2H, CH_2), 4.32-4.4.38 (q, 1H, 3′-H), 4.16-4.22 (q, 1H, 4′-H), 3.75 (s, 6H, OCH_3), 3.28-3.5 (m, 2H, 5′-H), 3.04-3.12 (t, 2H, CH_2), 2.71-2.75 (d, 1H, 3′-OH), 0.80 (s, 9H, $(CH_3)_3$), -0.07 (s, 3H, CH_3), -0.23 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.3, 164.9, 158.5, 156.5, 152.2, 151, 146.3, 145.1, 136.9, 136.1, 136.2, 134.4, 132.8, 130.3, 128.9-129.1 (d), 128.5, 127.1, 127, 121, 113, 87.2, 86.4, 84, 72.5, 71.8, 71.6, 63.9, 60.7, 55.4, 35.3, 25.8, 21.4 18, 14.5, -4.78, -4.88 HRMS: calcd for $C_{52}H_{57}N_5O_8NaSi$ [MNa^+] 930.3874, obsd 930.3867.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-[(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphino]-2′-*O*-(*t*-butyldimethylsilyl)-N^6-benzoyl-8-phenylethoxyadenosine(13). Yield 82-86% silica gel TLC R_f 0.6 (2:3 hexane:ethyl acetate); ^{1}H NMR (300 MHz, $CDCl_3$): 8.66 (s, 1H, CONH-), 8.40-8.46 (d, 1H, 2-H), 7.96-8.02 (m, 2H, PhCO), 7.42-7.62 (m, 5H, Ph), 7.20-7.39 (m, 12H, Ph), 6.74-6.82 (m, 4H, Ph), 5.88-5.94 (t, 1H, 1′-H), 5.36-5.46 (m, 1H, 2′-H), 4.68-4.78 (m, 2H, OCH_2), 4.30-4.54 (m, 2H, 3′-H and 4′-H), 3.82-4.02 (m, 1H, 5′-H), 3.76 (s, 6H, OCH_3), 3.51-3.70 (m, 4H, CH_2CN, 2CHN), 3.24-3.34 (m, 1H, 5′-H), 3.09-3.16 (t, 2H, CH_2), 2.51-2.70 (m, 2H, P-O-CH_2), 1.08-1.32 (m, 12H, $4CH_3$), 0.72-0.77 (d, 9H, $(CH_3)_3$), (-0.09)-(-0.04) (d, 3H, CH_3), (-0.33)-(-0.28) (d, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.5, 158.6, 156.8, 155.9, 152.4, 146.4, 145.0, 144.9, 137, 136, 134.5, 132.8, 130.4, 129.3, 129, 128.6, 128, 127, 117.6, 113.3, 98.8, 87.0, 86.5, 83.9, 73, 71.2, 63.5, 59.0-59.4 (d), 55.5, 43.5-43.8 (d), 35.5, 25.8, 24.7-25.2 (m), 21.4, 20.7, 18.2, 14.5, -4.4, -5.0. ^{31}P NMR (CD_2Cl_2): 149.0, 151.4. HRMS: calcd for $C_{61}H_{74}N_7O_9NaSiP$ [MNa^+] 1130.4953, obsd 1130.4973.

5′,3′-*O*-Bis(*t*-butylsilyl)-2′-*O*-(*t*-butyldimethylsilyl)-8-cyclohexylethoxyadenosine (14). Yield 65%; silica gel TLC R_f 0.49 (2:3 hexane:ethyl acetate); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.20 (s, 1H, 2-H), 5.89 (d, 1H, 1′-H), 5.34(s, 2H, NH_2), 4.92-4.97 (m, 1H, 2′-H), 4.68-4.74 (d, 1H, 3′-H), 4.47-4.61 (m, 2H, OCH2), 4.34-4.44 (m, 1H, 4′-H), 3.98-4.12 (m, 2H, 5′-H), 1.16-1.82 (m, 13H, $C_6H_{11}CH_2$), 1.02-1.16 (d, 18H, $(CH_3)_3$), 0.89 (s, 9H, $(CH_3)_3$), 0.04-.12 (d, 6H, $2CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 154.4, 153.2, 151.4, 149.6, 116.3, 90.4, 75.4, 74.6-74.8, 67.2, 67.8, 36.4, 33.3, 27.8, 27.3, 26.6, 26.3, 26.1, 22.9, 20.5, 18.6, -4.3, -4.9. HRMS: calcd for $C_{32}H_{58}N_5O_5Si_2$ [MH^+] 648.3977, obsd 648.4002.

5′,3′-*O*-Bis(*t*-butylsilyl)-2′-*O*-(*t*-butyldimethylsilyl)-N^6-benzoyl-8-cyclohexylethoxyadenosine (15). Yield 84-92%; silica gel TLC R_f 0.73 (2:3 hexane:ethyl acetate); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.76-8.82 (bs, 1H, CONH-), 8.59 (s, 1H, 2-H), 7.95-8.02 (m, 2H, PhCO), 7.48-7.62 (m, 3H, PhCO) 5.91-5.95 (d, 1H, 1′-H), 4.88-4.97 (q, 1H, 2′-H), 4.70-4.75 (d, 1H, 3′-H), 4.53-4.69 (m, 2H, OCH2), 4.35-4.43 (q, 1H, 4′-H), 4.98-4.14 (m, 2H, 5′-H), 1.16-1.82 (m, 13H, $C_6H_{11}CH_2$), 1.13 (s, 9H, $(CH_3)_3$), 1.05 (s, 9H, $(CH_3)_3$), 0.89 (s, 9H, $(CH_3)_3$), 0.06-0.14, (d, 6H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 165, 156.1, 152.1, 150.5, 146, 134.2, 132.7, 129.0, 128.0, 121.0, 90.5, 75.4, 74.8, 70.0, 67.7, 36.3, 33.3, 27.8, 27.3, 26.6, 26.3, 26.1, 22.9, 20.6, 18.7, -4.1, -5.0. HRMS: $C_{39}H_{61}N_5O_6NaSi_2$ [MNa^+] 774.4058, obsd 774.4078.

2′-*O*-*t*-Butyldimethylsilyl-N^6-benzoyl-8-cyclohexylethoxyadenosine(16). Yield 87-90%; silica gel TLC R_f 0.16 (2:3 hexane:ethyl acetate); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.79 (s, 1H, CONH-), 8.64 (s, 1H, 2-H), 7.94-8.00 (m, 2H, PhCO), 7.47-7.60 (m, 3H, PhCO), 6.04-6.12 (d, 1H, 5′-OH), 5.88-5.92 (d, 1H, 1′-H), 5.08-5.16 (q, 1H, 2′-H), 4.48-4.64 (m, 2H, OCH2), 4.30-4.36 (m, 2H, 3′-H and 4′-H), 3.88-3.98 (d, 1H, 5′-H), 3.67-3.78 (t, 1H, 5′-H), 2.90 (s, 1H, 3′-OH), 0.9-1.80 (m, 13H, $C_6H_{11}CH_2$), 0.80 (s, 9H, $(CH_3)_3$), -0.14 (s, 3H, CH_3), -0.37 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.7, 156.6, 151.0, 150.4, 147.0, 134.3, 132.9, 129.0, 127.0, 120.9, 88.0, 87.4, 73.9, 73.0, 70.2, 63.7, 36.3, 34.5, 33.4, 26.6, 26.3, 25.7, 18.0, -5.0, -5.2. HRMS: calcd for $C_{31}H_{46}N_5O_6Si$ [MH^+] 612.3217, obsd 612.3228.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*t*-butyldimethylsilyl)-N^6-benzoyl-8-cyclohexylethoxyadenosine (17). Yield 74-78%; silica gel TLC R_f 0.59 (2:3 hexane:ethyl acetate); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.70 (s, 1H, CONH-), 8.46 (s, 1H, 2-H), 7.96-

8.02 (m, 2H, PhCO), 7.14-7.62 (m, 12H, Ph), 6.72-6.80 (dd, 4H, Ph), 5.88-5.93 (d, 1H, 1′-H), 5.33-5.39 (t, 1H, 2′-H), 4.53-4.62 (t, 2H, OCH2), 4.38-4.44 (q, 1H, 3′-H), 4.16-4.23 (q, 1H, 4′-H), 3.76 (s, 6H, OCH_3), 3.30-3.50 (m, 2H, 5′-H), 2.73-2.77 (d, 1H, 3′-OH), 0.92-1.80 (m, 13H, $C_6H_{11}CH_2$), 0.84 (s, 9H, $(CH_3)_3$), -0.02 (s, 3H, CH_3), -0.2 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.8, 158.6, 156.8, 152.0, 150.8, 146.3, 145.0, 136.3, 136.2, 134.5, 132.7, 130-130.4 (d), 129.1, 128.5, 127.9, 126.9, 120.5, 113.2, 87.3, 86.4, 84.1, 72.7, 71.7, 70.0, 63.9, 55.4, 36.3, 34.5, 33.4, 33.3, 26.8, 26.4, 25.8, 18.1, -4.7, -4.8 HRMS: calcd for $C_{52}H_{63}N_5O_8NaSi$ [MNa^+] 936.4344, obsd 936.4332.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-[(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphino]-2′-*O*-(*t*-butyldimethylsilyl)-N^6-benzoyl-8-cyclohexylethoxyadenosine (18). Yield 78-85%; silica gel TLC R_f 0.59 (2:3 hexane:ethyl acetate); ^{1}H NMR (300 MHz, $CDCl_3$): 8.64-8.72 (d, 1H, CONH-), 8.35-8.44 (t, 1H, 2-H), 7.96-8.02 (m, 2H, PhCO), 7.16-7.62 (m, 12H, Ph), 6.74-6.82 (m, 4H, Ph), 5.86-5.94 (t, 1H, 1′-H), 5.35-5.48 (m, 1H, 2′-H), 4.53-4.62 (t, 2H, OCH2), 4.44-4.53 (m, 1H, 3′-H), 4.24-4.40 (m, 1H, 4′-H), 3.88-4.0 (m, 1H, 5′-H), 3.77 (s, 6H, OCH_3), 3.51-3.70 (m, 4H, CH_2CN, 2CHN), 3.24-3.38 (m, 1H, 5′-H), 2.54-2.74 (m, 2H, P-O-CH_2), 0.84-1.80 (m, 13H, $C_6H_{11}CH_2$; m, 12H, $4CH_3$), 0.72-0.80 (d, 9H, $(CH_3)_3$), (-0.08)-(-0.04) (t, 3H, CH_3), (-0.30)-(-0.26) (d, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.8, 158.6, 150.7, 145.0, 136.2, 132.8, 130.4, 129.1, 128.5, 128.0, 127.0, 120.1, 113.2, 86.4-87.0 (q), 83.0, 70.0, 63.8, 59.0, 59.4, 55.3, 53.5, 43-43.4 (d), 36.3, 34.5, 33.2, 33.4, 26.6, 26.4, 25.8, 24.6-25.1 (m), 20.5-20.8, 18.1, -4.3, -4.9. ^{31}P NMR (CD_2Cl_2): 148.9, 151.5. HRMS: calcd for $C_{61}H_{80}N_7O_9NaSiP$ [MNa^+] 1136.5422, obsd 1136.5436.

Design of the caspase 2 siRNA and synthesis of the modified guide strands. Caspase 2 siRNA (A:U) and the corresponding negative control siRNA (scrambled sequence) was designed using Ambion's siRNA designing tool and checked for sequence similarity using nucleotide Basic Local Alignment Search Tool (BLAST). No significant similarity was found with other genes. To analyze caspase 2 expression levels quickly and reliably, a plasmid-based dual luciferase assay system (psiCHECK2 vector) was employed. A fragment of the caspase 2 mRNA sequence was inserted into the vector, and the resulting reporter plasmid was used to evaluate caspase 2 mRNA knockdown.

8-AlkoxyA phosphoramidites was subsequently coupled into various positions of the guide strand of the caspase 2 siRNA. All siRNA oligonucleotides, modified and unmodified, were synthesized by standard solid-phase RNA synthesis on DNA columns in the DNA/Peptide Core Facility of the University of Utah using an Applied Biosystems (Model 394) DNA/RNA synthesizer. The positions chosen for single substitution were 4, 6, 10 and 15; doubly modified guide strands had modifications at (6,10), (6,15) and (10, 15) positions (Figure 2.10).

Synthesis and purification of siRNAs. After synthesis, the 21-mer RNA oligonucleotides were cleaved from the column and deprotected using methanolic ammonia for 24 h at room temperature, and the 2′-OTBDMS group was deprotected by using TEA•3HF overnight at room temperature. The oligomers were then dialyzed at 4 °C for 6 h, purified by semi-preparative ion-exchange HPLC, dialyzed at 4 °C again to get rid of excess salt, lyophilized and stored at -20 °C under dry conditions.

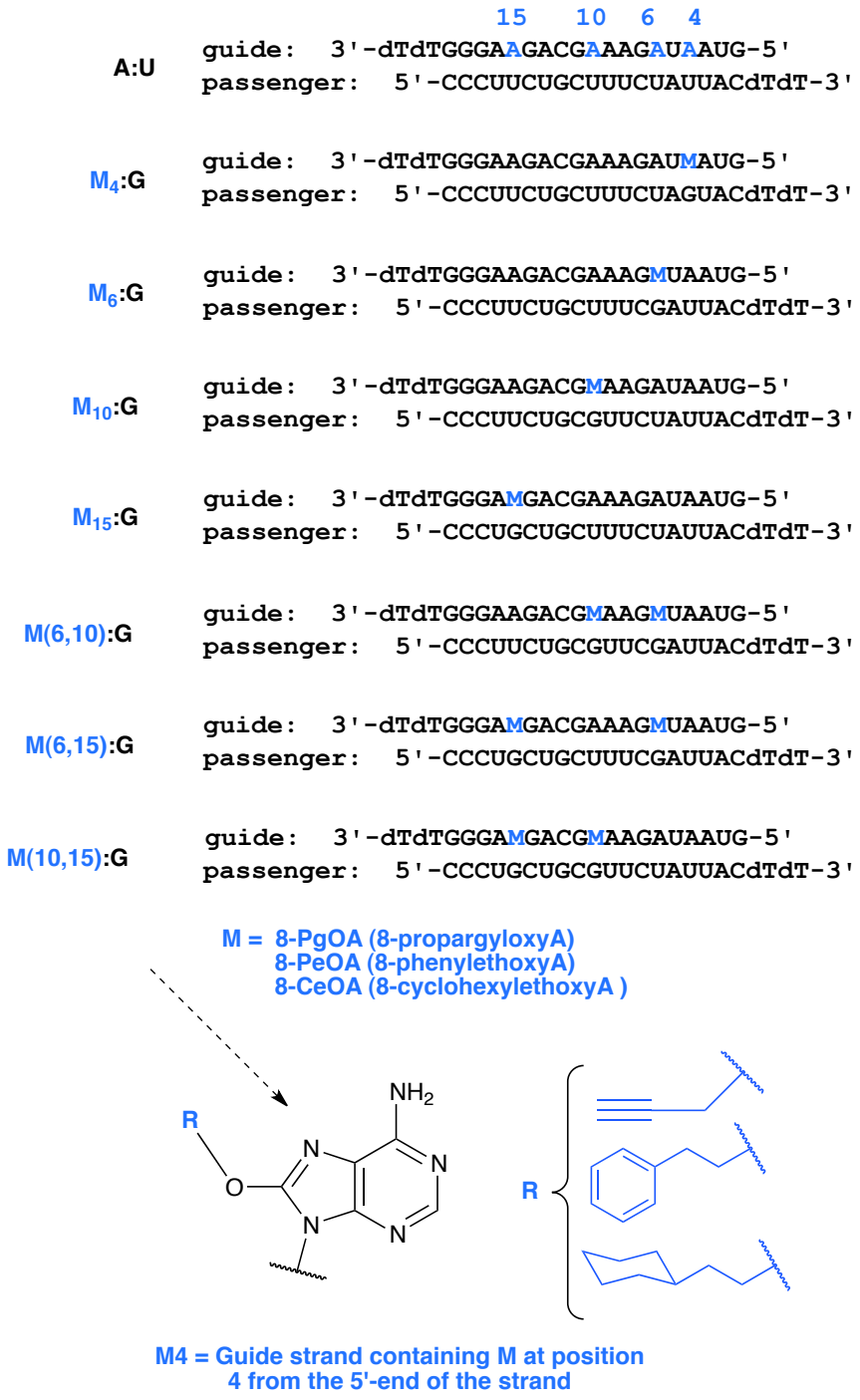


Figure 2.10. SiRNA sequences used. A:U represents unmodified siRNA and modified (denoted by M:G) sequences have the standard A:U base pair replaced by 8-alkoxyA(M):G in one or two positions in the siRNAs.

ESI-MS characterization of the modified guide strands. Two nanomoles of each modified guide strands were extensively dialyzed for 2 days at 4 °C in ammonium acetate solution to get rid of excess sodium ions. The final dialysis was carried out in water. The oligomers were then lyophilized, and dissolved in 1:1 isopropanol:water and analyzed by electrospray ionization mass spectrometry (ESI-MS) in negative ion mode. The results are furnished in the Table 2.1.

Results and discussion

Purines can exist preferentially as the *anti* conformer and participate in canonical Watson-Crick H-bonding with their complementary bases. However, there is evidence of transient *syn* adenosines in duplex RNA (*24*). Also, in the context of AG mismatches, there is evidence of *syn* A in double-stranded DNA (*25*). It was observed that the *syn/anti* equilibrium of purines could be shifted more towards the *syn* conformation by introducing a substitution at position 8; this substituent experiences a steric clash with the 5′ hydrogen atoms of the ribose backbone as well as with the 4′ oxygen, facilitating the *syn* conformation to a greater extent than the unmodified purine.

Indeed, 8-BrdG, 8-O-dA, 8-O-dG, 8-BrdA and 8-MeOdA are known to exist in mixtures of *syn*/*anti* equilibrium (*26-29*). Here, the unusual RNA base modification 8-alkoxyA was chosen as a potential ‘conformational switch’ in the guide strand of a caspase 2 siRNA. 8-AlkoxyA, being a Janus-faced base, can expose the Watson-Crick face in the *anti* conformation, as well as the Hoogsteen face in the *syn* conformation; thus *anti* 8-alkoxyA can base-pair canonically with U and *syn* 8-alkoxyA has potential to form H-bonds with *anti* G (Figure 2.6).

Table 2.1. ESI-MS of the modified guide strands.

Guide strand	Guide strand sequences	Calculated mass	Experimental mass
Pg4	3′- ttGGGAAGACGAAAGAU**Pg**AUG - 5′	6897.4	6896.9
Pg6	3′- ttGGGAAGACGAAAG**Pg**UAAUG - 5′	6897.4	6896.8
Pg10	3′- ttGGGAAGACG**Pg**AAGAUAAUG - 5′	6897.4	6896.9
Pg15	3′- ttGGGA**Pg**GACGAAAGAUAAUG - 5′	6897.4	6896.9
Pg6,10	3′- ttGGGAAGACG**Pg**AAG**Pg**UAAUG - 5′	6973.4 (Na^+ salt)	6972.5
Pg6,15	3′- ttGGGA**Pg**GACGAAAG**Pg**UAAUG- 5′	6973.4 (Na^+ salt)	6972.8
Pg10,15	3′- ttGGGA**Pg**GACG**Pg**AAGAUAAUG - 5′	6973.4 (Na^+ salt)	6972.8
Pe4	3′- ttGGGAAGACGAAAGAU**Pe**AUG - 5′	6963.6	6964.8
Pe6	3′- ttGGGAAGACGAAAG**Pe**UAAUG - 5′	6963.6	6964.8
Pe10	3′- ttGGGAAGACG**Pe**AAGAUAAUG - 5′	6963.6	6964.0
Pe15	3′- ttGGGA**Pe**GACGAAAGAUAAUG - 5′	6963.6	6964.8
Pe6,10	3′- ttGGGAAGACG**Pe**AAG**Pe**UAAUG - 5′	7083.8	7083.6
Pe6,15	3′- ttGGGA**Pe**GACGAAAG**Pe**UAAUG - 5′	7083.8	7083.7
Pe10,15	3′- ttGGGA**Pe**GACG**Pe**AAGAUAAUG - 5′	7083.8	7083.1
Ce4	3′- ttGGGAAGACGAAAGAU**Ce**AUG - 5′	6969.6	6969.6
Ce6	3′- ttGGGAAGACGAAAG**Ce**UAAUG - 5′	6969.6	6969.6
Ce10	3′- ttGGGAAGACG**Ce**AAGAUAAUG - 5′	6969.6	6970.4
Ce15	3′- ttGGGA**Ce**GACGAAAGAUAAUG - 5′	6969.6	6970.4
Ce6,10	3′- ttGGGAAGACG**Ce**AAG**Ce**UAAUG - 5′	7095.8	7095.5
Ce6,15	3′- ttGGGA**Ce**GACGAAAG**Ce**UAAUG - 5′	7095.8	7095.0
Ce10,15	3′- ttGGGA**Ce**GACG**Ce**AAGAUAAUG - 5′	7095.8	7095.0

8-AlkoxyA phosphoramidites were synthesized in good yields in multiple steps from A. 8-AlkoxyA was synthesized from the corresponding bromo intermediate through aromatic nucleophilic substitution of the Br group with the alkoxy group. The initial bromination of adenosine is a literature procedure. Usually, three- to four-fold excess of bromine afforded good yields (75-82.5%) of the 8-bromoadenosine (**1**).

A major problem in the standard synthesis of ribo-phosphoramidites is the selective protection of the 2′-OH group over the 3′-OH. The Ag^+ ion was reported to improve the yield of the 2′-O-protected intermediate; but even Ag^+ ion cannot always minimize the unavoidable reaction at the 3′-OH (*30*). As a consequence, the overall yield of the desired phosphoramidite is significantly reduced. To accomplish a better overall yield, we decided to use a bidentate hydroxyl protecting group, di-*t*-butylsilyl ditriflate (DTBSDT) (*23*), that will protect the 5′- and 3′-OH simultaneously, leaving the 2′-OH ready to be protected by *t*-butyldimethylsilyl group. These consecutive steps were basically a one-pot reaction; so no tedious separation was required. Additionally, the reaction was almost quantitative with 98% product (**3**) yield.

Synthesis of the propargyloxy derivative (**4**) was accomplished by the *in-situ*-generated 8-propargyloxylithium reagent; the reagent was synthesized by the reaction of n-BuLi with excess anhydrous propargyl alcohol in THF. This reaction was also very efficient and the yield was 94%. In spite of using such a harsh condition, no detectable side reaction was observed.

The exocyclic NH_2 of **4** was protected using the benzoyl group. When benzoyl chloride was used as the benzoylation reagent in pyridine, dibenzoyl derivative is also formed. Brief ammonia (in methanol) treatment was necessary to convert the dibenzoyl

derivative to the monobenzoyl one. Selective deproection of the 5′- and 3′-hydroxyls was carried out by using a mild fluoride reagent, HF-pyridine. However, at 25 °C both the DTBSDT and the TBDMS protecting groups are deprotected (*23*), By carrying out the reaction at –10 °C for 2-2.5 h, 2′-OTBDMS protection remains intact.

Following standard solid phase oligonucleotide synthesis, the 5′-OH was protected by the DMT group. Literature yields of this reaction for many modified nucleosides are always low to moderate. In this case the yield was low (<50%) initially; however changing the reaction conditions slightly afforded 70-80% yields. Addition of DMT-Cl to the solution (in pyridine) of **6** at low temperature (0 °C) and gradually warming up the solution was essential to obtain higher yields. Addition of one more equivalent of DMT-Cl after 8 h of the first addition also improved yield of the DMT derivative. The propargyloxy phosphoramidite (**7**) was synthesized in good yield using standard phosphoramidite reagent. The phosphoramidite is stable for 48 h in methylene chloride or THF; if lyophilized, it can be stored at -20 °C for a year without degradation.

The 8-benzyloxy derivative, synthesized from **3,** was partially degraded during chromatographic separation (slightly acidic pH) of the nucleoside analog (Figure 2.11). We were doubtful whether this modification could survive through the strongly acidic solid phase synthetic steps and the RNA deprotection steps. Hence we decided to explore the phenylethoxy and cyclohexylethoxy modifications in this context. Phosphoramidites containing these two modifications were synthesized following analogous procedures. Coupling efficiency of the modified phosphoramidites were slightly inferior to natural ribonucleoside phosphoramidites probably due to the presence of sterically demanding alkoxy groups at position 8 of the adenine ring.

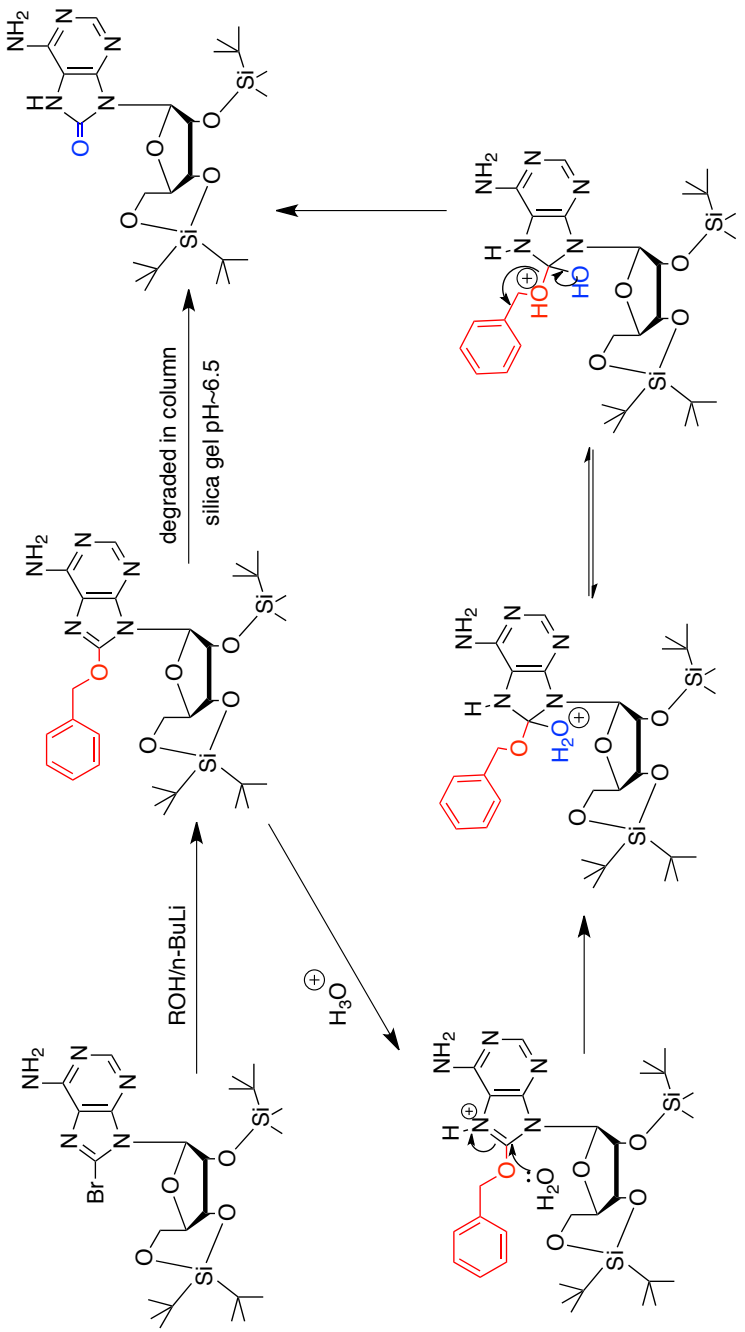


Figure 2.11. Degradation of the 8-benzyloxyadenosine derivative.

Mass spectral analysis of the 8-alkoxyadenosine containing, guide strands confirmed their solid phase incorporation into oligoribonucleotides and stability under normal RNA deprotection conditions (Table 2.1). However, these modified nucleosides start degrading at high pH (>8.5); under this condition, the alkoxy group is converted to oxo group, probably through an aromatic nucleophilic substitution mechanism. Hydroxide anion substitutes the alkoxy group and the resultant 8-hydroxyA tautomerizes to the more stable 8-oxoA.

Modifications were placed in the guide strand because, not too many successful base modifications were known, to date, in the guide strand (*21, 31*). Also guide base modifications can interact with the RNAi machinery even when the passenger strand is stripped of the RISC complex; hence intricate mechanistic details can be explored with appropriate modifications. This is certainly not possible with the passenger strand modifications.

Propargyl, phenethyl and cyclohexylethyl groups were chosen based on the size and shape of the alkyl groups. The choice of alkyl groups was also considered in terms of prevention of siRNA-PKR interaction as well as maintenance of target mRNA knockdown. Methyl or ethyl groups might be too small to have any significant impact on the siRNA-protein interactions. However, it was also anticipated that too large a group in the guide strand might reduce significantly or even completely abolish the RNAi efficacy.

Hence, we explored the series with the propargyloxy modification, which was expected to retain the RNAi efficacy, as well as prohibit the unwanted protein binding to some extent. The phenylethoxy group was chosen as the most promising group in the

guide strand, which could serve both purposes. We were optimistic that cyclohexylethyl modifications will prevent dsRBM-containing proteins from binding siRNAs, but were interested to explore whether such a large modification might also compromise RNAi efficacy.

Earlier, Beal and coworkers reported that minor-groove base modifications in the passenger strand could prevent PKR and ADAR1 binding when modifications were placed at 6, 9, 11 and 14 or at proximate positions (Figure 2.8) (*18*). 8-AlkoxyA was substituted for A in single or multiple positions of the guide strands; so, the presence of an A in those positions or at nearby positions was taken into account while making the selection.

Positions of modifications were based on significance of the positions for mediating the gene silencing, as well as preventing the off-pathway protein binding. Positions 4 and 6 (from the 5′-end of the guide strand) are important positions in the seed region of siRNA; position 10 is just next to the cleavage site. We were interested to know the effect of moderate (propargyloxy) or large (phenylethoxy and cyclohexylethoxy) modifications in the minor and major grooves at these sites. Position 15 is located towards the 3′-terminal of the guide strand and this part of the siRNA is known to tolerate a large number of modifications irrespective of the size and shape.

Conclusion

Chemical modifications impart many novel properties into siRNAs that are not achievable in unmodified siRNAs. Ribose modifications such as UNA helped reduce off-target effects and prevented sequence-dependent off-target effects. 2′-F Modifications helped achieve target specificity. Off-pathway protein interactions with the siRNA are

still one of the major limitations of the siRNA-based RNAi therapeutics. These undesired interactions reduce the effective concentration of the siRNA *in vivo,* instigate interferon-mediated immunostimulation, and globally affect the expression of many unrelated genes. Therefore, these interactions need to be successfully prevented to maximize the therapeutic efficacy of the siRNA.

The Beal laboratory and the Burrows laboratory are systematically exploring the possibilities of nucleobase modifications to address off-pathway protein binding with the siRNA. Minor grove modifications of purines, e.g., guanine and 2-aminopurine, in the passenger strand of a caspase 2 siRNA blocked such off-pathway interactions. Kannan et al. reported 'switchable' N^2 alkylated 2′-deoxy-7,8-dihydro-8-oxoguanosine in the guide strand of an siRNA to reduce the siRNA-PKR interaction, while not compromising the RNAi efficacy by much. However, 2′-deoxy-7,8-dihydro-8-oxoguanosine lesions are known to be immunostimulatory; therefore development of alternative base 'switches' is essential for effective substitution in the guide strand.

8-Substituted adenosines are known to exist in a mixture of *syn* and *anti* conformation around the glycosidic bond and can function as Janus-faced bases. Thus 8-alkoxyA can also exist as *syn* or *anti* conformer depending on the base-pairing partner. In the *syn* conformation, 8-alkoxyA can expose its Hoogsteen face to the complementary base and hence can base-pair with *anti* G; on the other hand, *anti* 8-alkoxyA can utilize its natural Watson-Crick face to form hydrogen bonding with *anti* U. These 'switchable' bases have the potential to prevent off-pathway interaction with dsRBM containing proteins in the *syn* conformation, as well as maintain RNAi efficacy as the *anti* conformer.

In this study, 8-alkoxyA phosphoramidites were synthesized and incorporated into the guide strand of a caspase 2 siRNA. Three different alkyl groups were used for the alkoxy modifications: propargyl (Pg), phenethyl (Pe) and cyclohexylethyl (Ce). Yields were good for both 8-propargyloxyA (8-PgOA) and 8-phenylethoxyA (8-PeOA) phosphoramidites; however the 8-cyclohexylethoxyA (8-CeOA) derivative afforded moderate yield. Modified phosphoramidites were incorporated into the guide strand of a caspase 2 siRNA through the solid phase oligonucleotide synthesis. Four singly modified and three doubly modified guide strands were synthesized to explore the effect of modifications on RNAi efficacy and off-pathway protein binding. Modified siRNA strands were stable under normal RNA deprotection conditions. ESI-MS analysis of the HPLC-purified RNA oligomers confirmed the presence of the modified bases in the RNA oligomers.

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CHAPTER 3

THERMAL ANALYSIS AND BIOLOGICAL EVALUATION OF THE MODIFIED SIRNAS

Introduction

Thermal denaturation studies of duplex nucleic acid oligomers is important in many biological applications such as in qualitative and quantitative PCR, hybridization probes, sequencing, aptamer and ribozyme designing and in the antisense and RNAi technologies. If the thermal stability of a duplex nucleic acid is very high, it will not unwind easily and the desired functions of the oligonucleotide will be affected; contrarily, instability of the duplex will pose similar problems. In addition, poor thermal stability will decrease the solution and nuclease stability of RNAs, because RNA is many times more stable as a duplex than as single strands.

The melting temperature (T_m) of a duplex oligonucleotide is defined as the temperature at which 50% of the duplexes exist as single strands. This dual state model holds good for most short oligonucleotides, but for long oligonucleotides complex transition patterns might be observed.

Importance of thermal denaturation analysis of the siRNA. Thermal analysis of the siRNA is a crucial step in designing potent and specific RNAi therapeutics. For effective gene silencing through RNAi, optimal melting temperature of the siRNA is desired. Many factors can influence the T_m of an siRNA; some important parameters are

the sequence, siRNA concentration and salt concentration. Generally GC-rich sequences have higher T_m compared to AT-rich sequences; for optimal RNAi efficacy of the siRNA, 38-60% GC content is desirable. The T_m of an siRNA is also dependent on the nucleotide and salt concentration in the solution; the higher the concentration, the higher is the T_m. Hydrogen bonding interactions between the complementary bases and base stacking interactions from the nearest neighbors play crucial roles in determining the T_m of an siRNA.

Higher thermal stability of the siRNA duplex at the 3′-end of the guide strand compared with the 5′-end is desired for optimal RNAi efficacy. Therefore, many successful siRNAs have a GC-rich region towards the 3′-end of the guide strand.

Consequences of different chemical modifications on the melting temperature (T_m) of the siRNAs. Chemically modified siRNAs are now integral parts of the siRNA therapeutics; therefore, the effect of any modification on the thermal stability of the duplex is an important consideration as well. Backbone modifications have mixed effects on the thermal stability of the siRNA. Phosphorothioate (PS) (*1*) modifications slightly lower the T_m of an siRNA, whereas boranophosphate (BP) (*2, 3*) modifications elevate this parameter slightly. Small ribose modifications may elevate the T_m of the siRNA (*1, 4*); contrarily, large ones destabilize it and the extent of destabilization depends on the size of the substituted group (*5*). 2′-Deoxy modifications lower the T_m of the siRNAs slightly, and substitution of the 4′-O with S has enhanced the T_m. Locked nucleic acids, LNA and ENA (*1*) and 2′-F (*4*) modifications increases the T_m of the duplex by helping the modified nucleoside to attain the RNA-like ‘3′-endo’ conformation.

Base modifications bring about the greatest changes in the thermal stability of the siRNA. Modifications that disrupt hydrogen bonding between bases (e.g. in N^3-Me-U), drastically lower the thermal stability of the duplex, whereas those enhancing base pairing strength through additional hydrogen bond(s) (2,6-diaminopurine) increase the T_m (*6*). Placement of hydrophobic nucleoside mimetics in the siRNA, lower the thermal stability of the duplex (*7, 8*). The Kool group reported siRNA major groove modifications that can stabilize the duplex RNA more than the unmodified ones (*9*). RNA minor groove modifications have always lowered the T_m irrespective of the position and size of the modification and the nature of the base modified (*10-12*).

Influence of thermal stability on the RNAi efficacy. An siRNA needs to be sufficiently stable as a duplex to be able to knock down a specific mRNA; however no general trend was observed between the T_m of the siRNA and the RNAi efficacy. Rana and coworkers reported that strengthening the hydrogen bonding, thereby elevating the T_m of the siRNA, between the complementary nucleobases has a deleterious effect on the RNAi efficacy. Thus 2,6-diaminopurine, when used in place of A towards the 5′-end of the guide strand, makes the siRNA less effective (*6*). Their study implies that the thermal asymmetry of the siRNA is more important than its thermal stability; the 5′-end of the guide strand should always be less stable than the 3′-end.

This observation was further clarified by utilizing hydrophobic and non-hydrogen-bonding nucleobase isosteres in the seed region of the passenger strand of an siRNA. Modifications such as 2,4-difluorotoluene, hypoxanthine, 5-nitroindole, purine, and 2-aminopurine at various positions within the passenger strand, improved RNAi efficacy mainly due to thermal destabilization (by 1 to 12 °C) of the duplex (*13*). On the contrary,

ribose modifications, such as LNA, ENA (*1*) and 2′-F (*4*) substitutions, substantially raised the T_m without compromising the RNAi efficacy.

Chemical modifications modulate siRNA efficacy. Chemical modifications of the ribose moiety (*1, 4, 6, 14-28*) and the phosphodiester backbone (*1, 6, 18*) have improved siRNA stability (*1, 4*), ribonuclease resistance (*1, 14, 17, 19, 22*), potency (*14, 19, 27*) and specificity (*1, 29*). Numerous polymer- (*30-32*), lipid- (*33*), cholesterol- (*34, 35*), carbohydrate- (*30, 31*), peptide- (*36*) and small molecule-based (*34*) terminal modifications of the siRNA exhibited effective RNA interference and polymer- and cholesterol-based conjugates facilitated intracellular delivery of siRNA both *in vitro* and *in vivo* (*37*). Base modifications have been shown to enhance thermal stability (*9*) and nuclease stability (*7*); these also helped visualize intracellular trafficking (*38*) and reduced off-target effects (*10, 11, 39*). Recently, major groove substitution of modified purines in various positions of the guide strand have been reported; here also RNAi efficacy was found to be position dependent (*40*).

Off-pathway protein interactions with the siRNA. Along with the targeted delivery issue, off-target gene silencing and off-pathway protein interactions leading to immunostimulation are still the major limitations for rapid optimization of the siRNA therapeutics. Specific siRNA sequences can bind to toll-like receptors and lead to immunostimulation (*41-45*). In addition, sequence-independent off-pathway protein interactions involve binding of virtually any siRNA with double-stranded RNA binding motif (dsRBM) containing proteins, e.g., RNA-dependent protein kinase (PKR), adenosine deaminases (ADARs) and other intracellular proteins (*46, 47*). PKR activation leads to phosphorylation of eukaryotic Initiation factor 2, inhibition of translation

initiation and other antiviral signaling events (*48*). Although siRNAs are shorter than ligands that trigger high PKR activity, lower level activation of PKR has been observed with siRNAs *in vitro* and when transfected into certain cell types (*41*).

ADARs also bind dsRNA and have been shown to interact with substrates within the RNAi pathway (*49, 50*). The full-length isoform of ADAR1 (ADAR1p150) has been linked to decreased siRNA potency in mammalian cells, presumably due to the formation of high affinity siRNA•ADAR1p150 complexes within the cytoplasm (*51*). Additionally, ADAR has been shown to reduce RNAi efficiency in *Drosophila* cell culture (*50*). Therefore, prevention of the siRNA binding to dsRBM-containing proteins off the RNAi pathway is important for the potency and specificity of therapeutic RNAi.

Some nucleoside analogs have shown promise in preventing PKR binding, but no systematic study with modified sugars to address such sequence-independent induction of immunostimulation is available (*52*). On the other hand, altering the RNA minor groove through base modifications has shown potential in modulating siRNA properties and siRNA-protein interactions. The Beal laboratory initiated a systematic study on siRNA base modifications that could block off-pathway protein binding (*53*), and they have also shown that pendent minor groove modifications of guanines (*10*) and 2-aminopurines (*11*) in the passenger caspase 2 siRNA can successfully prevent siRNA-dsRBM interactions, while maintaining the RNAi efficacy.

Guide strand nucleobase modifications to prevent off-pathway protein binding. Base modifications in the guide strand are more challenging, since many modifications at crucial siRNA sites (e.g., in the seed region or at the cleavage site) will drastically reduce the silencing efficacy or might even completely abolish the knockdown

of the desired mRNA. In contrast, modifications at those sites might, in fact, turn out to be more exciting and lead to elucidation of crucial mechanisms in the RISC complex. For example, appropriate modifications in the minor or major groove of an siRNA can help explore the siRNA mechanisms in detail, and placement of designer modifications in those grooves can provide further novel insights into the siRNA-RISC interactions.

Kannan et al. have shown that N^2-alkylated 2′-deoxy-7,8-dihydro-8-oxoguanosine-containing siRNAs can prevent siRNA-PKR interaction significantly by placing a 'switchable' alkyl group in the minor groove of an siRNA during delivery; in the RISC, the modified siRNAs can flip the sterically encumbering group into the major groove and maintain good RNAi efficacy (*39*). However 8-oxoguanine-containing DNA oligomers are known to be inflammatory and immunostimulatory themselves (*54, 55*). Therefore, we chose to explore alternative purine modifications in the guide strand. Here we report the syntheses, thermal stability and biological activity of 8-alkoxyA-substituted siRNAs.

Janus-faced 8-alkoxyA nucleosides in the guide strand. 8-AlkoxyA-modified siRNAs were tested for their RNAi efficacy and ability to address off-pathway protein binding due to siRNA-PKR interactions. 8-Substituted adenosines and guanosines have been shown to exist in an equilibrium mixture of *syn/anti* conformers. In this work, 8-alkoxyAs are postulated to flip between *anti* and *syn* conformations depending on the base-pairing partner. In the natural *anti* conformation, 8-alkoxyA will base pair with U; whereas in the *syn* conformation, the Hoogsteen face of the nucleoside will be exposed for base pairing, and its best complement would be *anti* G (Figure 3.1). We propose that, during siRNA delivery, 8-alkoxyA in the guide strand (complementary to G in the passenger strand) would project the bulky alkoxy group into the minor groove of siRNA,

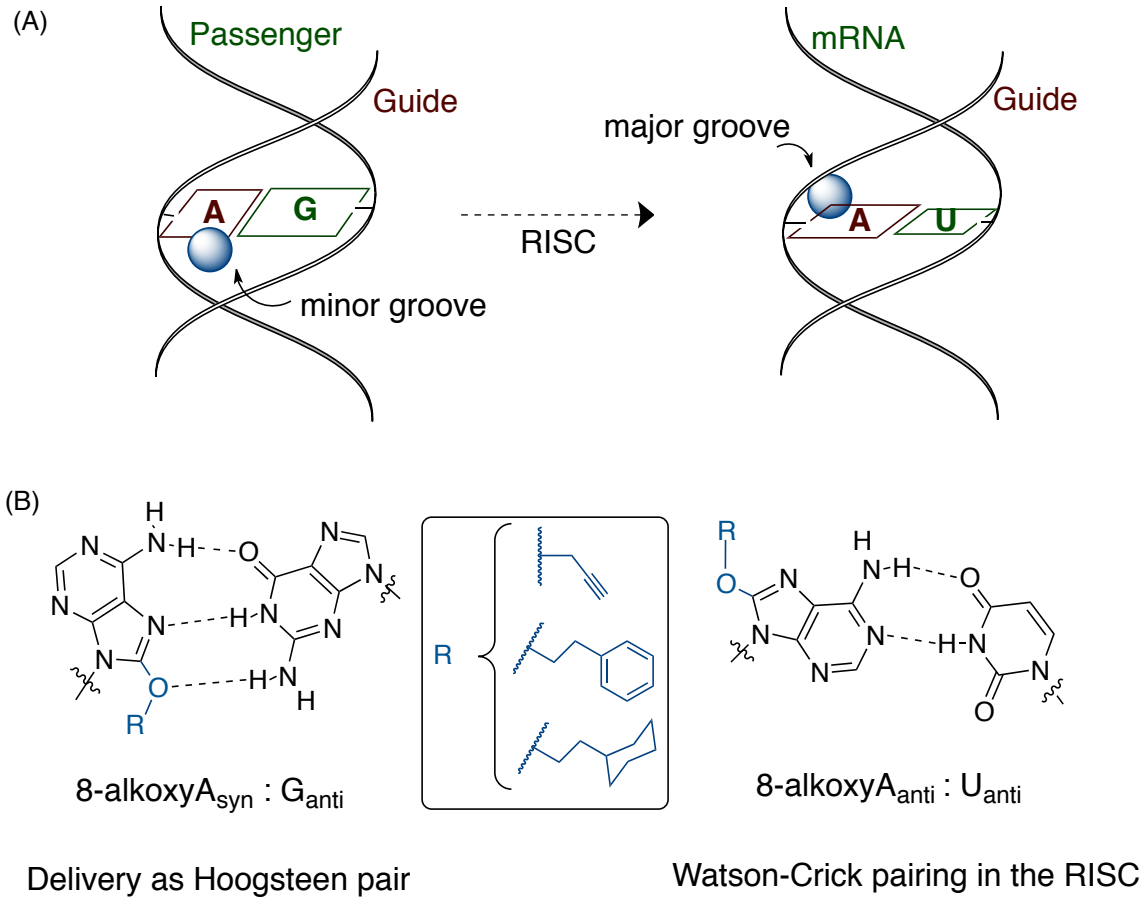


Figure 3.1. Proposed 8-AlkoxyA 'base switches'. (A) Cartoon showing flipping of a steric blockade from the minor to the major groove. (B) Proposed base pairs of the 8-alkoxyAs.

thereby preventing off-pathway protein binding. When the siRNA is recruited into the RISC assembly, the 8-alkoxyA in the guide siRNA would encounter U in the mRNA and would flip the bulky appendage into the major groove, thereby allowing necessary guide strand-mRNA-RISC assembly to form.

Alkyl groups chosen based on size and shape are propargyl (Pg), phenethyl (Pe) and cyclohexylethyl (Ce). The rationale behind the choices is that smaller groups (such as propargyl) might exhibit higher duplex stabilities and better mRNA knock downefficiencies whereas a larger group (cyclohexylethyl) might prevent immunostimulation to a greater extent, and a medium sized group (phenethyl) might serve both the purposes equally efficiently.

Our study suggests that the mRNA knock down ability of the singly modified siRNAs is quite similar to that of the unmodified positive control siRNA. Singly modified siRNA oligomers have also exhibited a reduced tendency to bind PKR. With additional alkoxy substitution, the efficiency of the siRNA is significantly reduced; therefore multiply modified siRNAs were not tested for off-pathway protein interactions.

Experimental

SiRNA annealing. SiRNA oligomers were dissolved in annealing buffer (100 mM NaCl and 10 mM TRIS, pH 7.1), and appropriate guide and passenger strands were mixed in equal amounts in an Eppendorf tube. The nucleotide mixtures were placed in a 90 °C water bath for 5 min, and the bath was gradually cooled to room temperature in about 2 h.

Thermal analysis of the modified siRNAs. Modified guide strands were hybridized against passenger strands containing both U and G (Tables 3.1 and 3.2). Melting temperature experiments of the hybridized duplexes were performed in a Beckman DU 650 spectrophotometer. In all experiments, the temperature was varied from 25 °C to 80 °C, and the rate of heating was 0.5 °C/min. Thermal denaturation was monitored at 260 nm. All experiments were performed with 1 nmol duplex RNA in siRNA annealing buffer (containing 10mM Tris, 100 mM NaCl, pH 7.1) in triplicate and normalized to appropriate blank controls. The reported T_m value was the average of 3 independent experiments, and error bars represent standard deviation from the average value. Experimental data were analyzed by 'two point average' method. 4 cuvettes were filled with 325 µL annealing buffer and were read as blanks. Then, 3 cuvettes were emptied, and 1 nmol siRNA duplex was added to the siRNA annealing buffer so that the total volume of the mixture became 975 µL. This mixture was equally distributed in those 3 cuvettes, and the fourth cuvette was loaded with 325 µL annealing buffer. Lids were closed tightly and temperature was varied from 25 to 80 °C.

Synthesis of the wild-type and mutant plasmids. The appropriate caspase 2 inserts (Figure 3.3) were introduced into the psiCHECK2 vector (Figure 3.2) using the multiple cloning region of the plasmid. One wild type and two mutant plasmids were synthesized. The plasmids were multiplied in *E. coli*, extracted by Qiagen mini plasmid extraction kit and sequenced at the Core Facility, University of Utah. Figure 3.4 furnishes a brief protocol for the synthesis of the recombinant plasmid.

Cell culture. HeLa cells were cultured in Dubelco's DMEM cell culture medium with 10% FBS and maintained under 5% CO_2 in an incubator. Corning 75 mL and 225

Table 3.1. Caspase 2 siRNAs containing 8-alkoxyA in the guide strands and U in the complementary passenger strands.

SiRNA	Guide strand sequences	Passenger strand sequences
A:U	5′-GUA**A**U**A**GAA**A**GCAG**A**AGGGtt-3′	5′-CCCU**U**CUGC**U**UUC**U**A**U**UACtt-3′
Pg4:U	5′-GUA**Pg**UAGAAAGCAGAAGGGtt-3′	5′-CCCUUCUGCUUUCUA**U**UACtt-3′
Pg6:U	5′-GUAAU**Pg**GAAAGCAGAAGGGtt-3′	5′-CCCUUCUGCUUUC**U**AUUACtt-3′
Pg10:U	5′-GUAAUAGAA**Pg**GCAGAAGGGtt-3′	5′-CCCUUCUGC**U**UUCUAUUACtt-3′
Pg15:U	5′-GUAAUAGAAAGCAG**Pg**AGGGtt-3′	5′-CCCU**U**CUGCUUUCUAUUACtt-3′
Pg6,10:U	5′-GUAAU**Pg**GAA**Pg**GCAGAAGGGtt-3′	5′-CCCUUCUGC**U**UUC**U**AUUACtt-3′
Pg6,15:U	5′-GUAAU**Pg**GAAAGCAG**Pg**AGGGtt-3′	5′-CCCU**U**CUGCUUUC**U**AUUACtt-3′
Pg10,15:U	5′-GUAAUAGAA**Pg**GCAG**Pg**AGGGtt-3′	5′-CCCU**U**CUGC**U**UUCUAUUACtt-3′
Pe4:U	5′-GUA**Pe**UAGAAAGCAGAAGGGtt-3′	5′-CCCUUCUGCUUUCUA**U**UACtt-3′
Pe6:U	5′-GUAAU**Pe**GAAAGCAGAAGGGtt-3′	5′-CCCUUCUGCUUUC**U**AUUACtt-3′
Pe10:U	5′-GUAAUAGAA**Pe**GCAGAAGGGtt-3′	5′-CCCUUCUGC**U**UUCUAUUACtt-3′
Pe15:U	5′-GUAAUAGAAAGCAG**Pe**AGGGtt-3′	5′-CCCU**U**CUGCUUUCUAUUACtt-3′
Pe6,10:U	5′-GUAAU**Pe**GAA**Pe**GCAGAAGGGtt-3′	5′-CCCUUCUGC**U**UUC**U**AUUACtt-3′
Pe6,15:U	5′-GUAAU**Pe**GAAAGCAG**Pe**AGGGtt-3′	5′-CCCU**U**CUGCUUUC**U**AUUACtt-3′
Pe10,15:U	5′-GUAAUAGAA**Pe**GCAG**Pe**AGGGtt-3′	5′-CCCU**U**CUGC**U**UUCUAUUACtt-3′
Ce4:U	5′-GUA**Ce**UAGAAAGCAGAAGGGtt-3′	5′-CCCUUCUGCUUUCUA**U**UACtt-3′
Ce6:U	5′-GUAAU**Ce**GAAAGCAGAAGGGtt-3′	5′-CCCUUCUGCUUUC**U**AUUACtt-3′
Ce10:U	5′-GUAAUAGAA**Ce**GCAGAAGGGtt-3′	5′-CCCUUCUGC**U**UUCUAUUACtt-3′
Ce15:U	5′-GUAAUAGAAAGCAG**Ce**AGGGtt-3′	5′-CCCU**U**CUGCUUUCUAUUACtt-3′
Ce6,10:U	5′-GUAAU**Ce**GAA**Ce**GCAGAAGGGtt-3′	5′-CCCUUCUGC**U**UUC**U**AUUACtt-3′
Ce6,15:U	5′-GUAAU**Ce**GAAAGCAG**Ce**AGGGtt-3′	5′-CCCU**U**CUGCUUUC**U**AUUACtt-3′
Ce10,15:U	5′-GUAAUAGAA**Ce**GCAG**Ce**AGGGtt-3′	5′-CCCU**U**CUGC**U**UUCUAUUACtt-3′

Table 3.2. Caspase 2 siRNAs containing 8-alkoxyA in the guide strands and G in the complementary passenger strands.

SiRNA	Guide strand sequences	Passenger strand sequences
A:U	5′-GUA**A**U**A**GAA**A**GCAG**A**AGGGtt-3′	5′-CCCU**U**CUGC**U**UUC**U**A**U**UACtt-3′
Pg4:G	5′-GUA**Pg**UAGAAAGCAGAAGGGtt-3′	5′-CCCUUCUGCUUUCUA**G**UACtt-3′
Pg6:G	5′-GUAAU**Pg**GAAAGCAGAAGGGtt-3′	5′-CCCUUCUGCUUUC**G**AUUACtt-3′
Pg10:G	5′-GUAAUAGAA**Pg**GCAGAAGGGtt-3′	5′-CCCUUCUGC**G**UUCUAUUACtt-3′
Pg15:G	5′-GUAAUAGAAAGCAG**Pg**AGGGtt-3′	5′-CCCU**G**CUGCUUUCUAUUACtt-3′
Pg6,10:G	5′-GUAAU**Pg**GAA**Pg**GCAGAAGGGtt-3′	5′-CCCUUCUGC**G**UUC**G**AUUACtt-3′
Pg6,15:G	5′-GUAAU**Pg**GAAAGCAG**Pg**AGGGtt-3′	5′-CCCU**G**CUGCUUUC**G**AUUACtt-3′
Pg10,15:G	5′-GUAAUAGAA**Pg**GCAG**Pg**AGGGtt-3′	5′-CCCU**G**CUGC**G**UUCUAUUACtt-3′
Pe4:G	5′-GUA**Pe**UAGAAAGCAGAAGGGtt-3′	5′-CCCUUCUGCUUUCUA**G**UACtt-3′
Pe6:G	5′-GUAAU**Pe**GAAAGCAGAAGGGtt-3′	5′-CCCUUCUGCUUUC**G**AUUACtt-3′
Pe10:G	5′-GUAAUAGAA**Pe**GCAGAAGGGtt-3′	5′-CCCUUCUGC**G**UUCUAUUACtt-3′
Pe15:G	5′-GUAAUAGAAAGCAG**Pe**AGGGtt-3′	5′-CCCU**G**CUGCUUUCUAUUACtt-3′
Pe6,10:G	5′-GUAAU**Pe**GAA**Pe**GCAGAAGGGtt-3′	5′-CCCUUCUGC**G**UUC**G**AUUACtt-3′
Pe6,15:G	5′-GUAAU**Pe**GAAAGCAG**Pe**AGGGtt-3′	5′-CCCU**G**CUGCUUUC**G**AUUACtt-3′
Pe10,15:G	5′-GUAAUAGAA**Pe**GCAG**Pe**AGGGtt-3′	5′-CCCU**G**CUGC**G**UUCUAUUACtt-3′
Ce4:G	5′-GUA**Ce**UAGAAAGCAGAAGGGtt-3′	5′-CCCUUCUGCUUUCUA**G**UACtt-3′
Ce6:G	5′-GUAAU**Ce**GAAAGCAGAAGGGtt-3′	5′-CCCUUCUGCUUUC**G**AUUACtt-3′
Ce10:G	5′-GUAAUAGAA**Ce**GCAGAAGGGtt-3′	5′-CCCUUCUGC**G**UUCUAUUACtt-3′
Ce15:G	5′-GUAAUAGAAAGCAG**Ce**AGGGtt-3′	5′-CCCU**G**CUGCUUUCUAUUACtt-3′
Ce6,10:G	5′-GUAAU**Ce**GAA**Ce**GCAGAAGGGtt-3′	5′-CCCUUCUGC**G**UUC**G**AUUACtt-3′
Ce6,15:G	5′-GUAAU**Ce**GAAAGCAG**Ce**AGGGtt-3′	5′-CCCU**G**CUGCUUUC**G**AUUACtt-3′
Ce10,15:G	5′-GUAAUAGAA**Ce**GCAG**Ce**AGGGtt-3′	5′-CCCU**G**CUGC**G**UUCUAUUACtt-3′

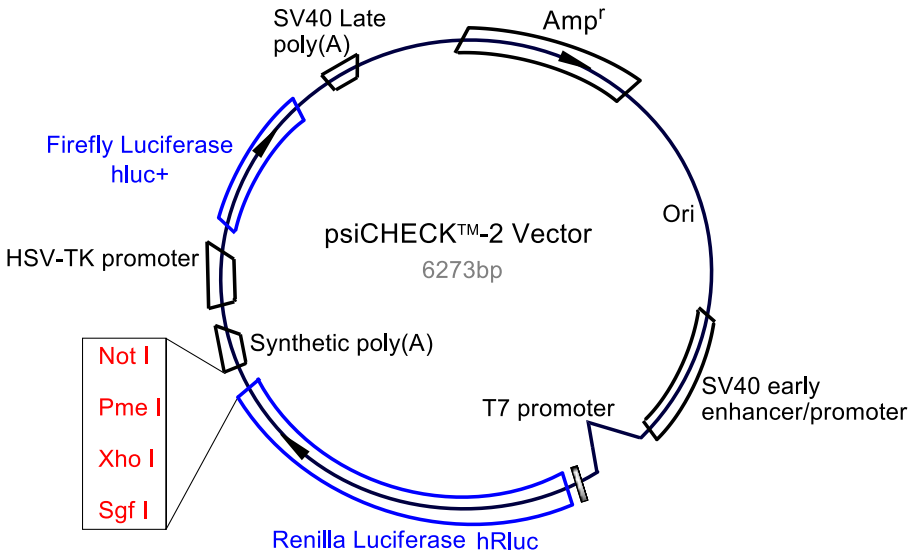


Figure 3.2. psiCHECK^{TM}-2 vector.

(A) 5´-TCGAGccccgAACCCTTCTGCTTTCTATTACtagaGC-3´
3´-CgggcTTGGGAAGACGAAAGATAATGatctCGCCGG-5´

(B) 5´-TCGAGccccgAACCCTTCTGCTTTCGATTACtagaGC-3´
3´-CgggcTTGGGAAGACGAAAGCTAATGatctCGCCGG-5´

(C) 5´-TCGAGccccgAACCCTTCTGCGTTCTATTACtagaGC-3´
3´-CgggcTTGGGAAGACGCAAGATAATGatctCGCCGG-5´

Figure 3.3. Caspase 2 inserts with Not I and Xho I restriction sites: (A) wild type (wt), (B) P6 mutant and (C) P10 mutant.

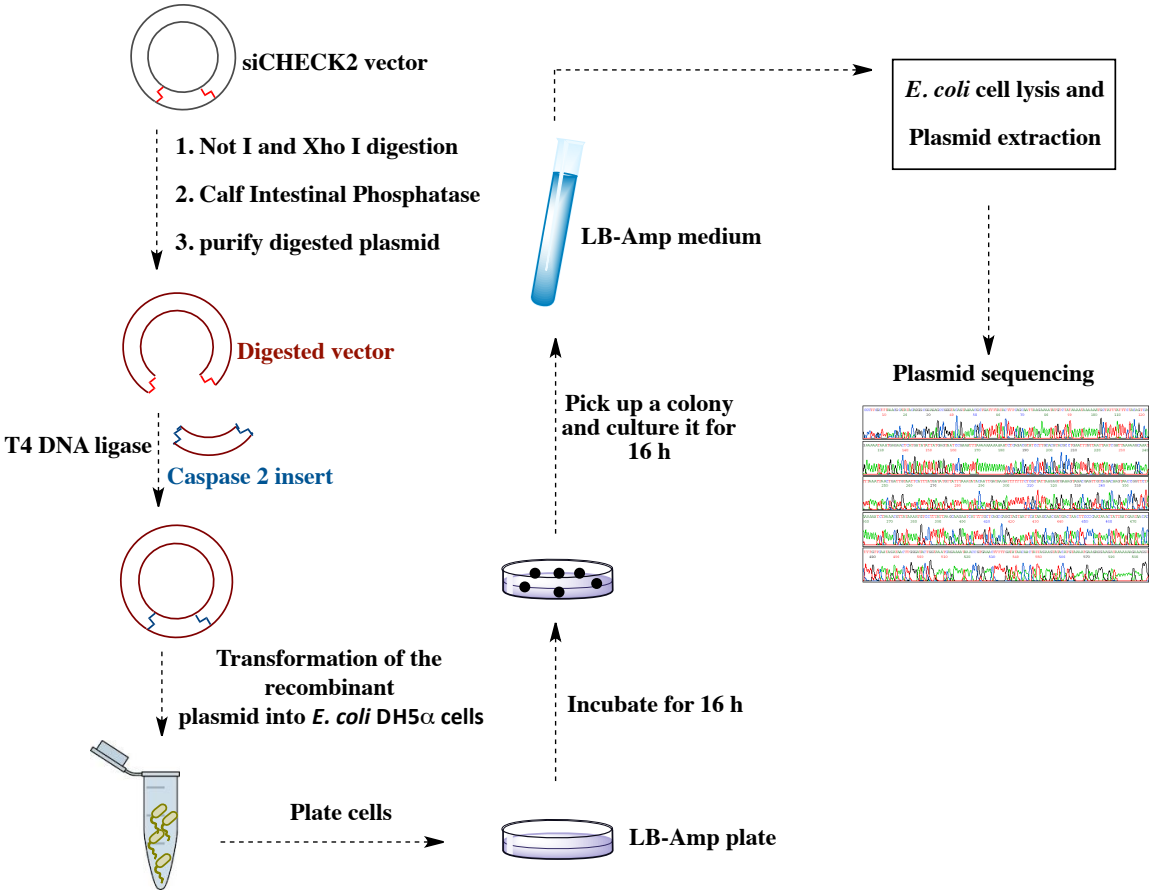


Figure 3.4. Synthesis of the recombinant plasmid.

mL cell culture flasks were used for growing cells. Water in the incubator pan was always autoclaved before use. The cells that were used in the experiments were between passage numbers 5 to 12. Cell confluence was kept between 50-70% for all RNAi assays.

The RNAi assay. During the day of the experiment, cells within the proper confluence level (~ 60%) were trypsinized and detached from the flask. Excess medium was added to inactivate trypsin. Cells were then centrifuged and re-suspended in fresh medium. Cells were counted manually by using a standard hemocytometer and diluted so that 6000 cells are present per 80 µL medium. Cells were kept in the incubator at 37 °C for about 45 min, while siRNA transfection complexes were being formed. siPORT™ NeoFX™ transfection agent was used as the siRNA delivery method in all cell culture studies. All siRNAs and plasmids were diluted in Opti-MEM® reduced serum medium, and siRNAs and plasmids were mixed together; similarly transfection agents were diluted in Opti-MEM® and transfection complexes were allowed to form. Then, siRNA-plasmid mixtures are added into diluted transfection agent solutions and mixed thoroughly by pipetting.

In all the experiments, 96-well plates were used and 20 µL siRNA-transfection complex was discharged into each well (Figure 3.5). The cell suspension was then taken out of the incubator and 80 µL of the cell suspension (containing 6000 cells) was added to each well. The plate was shaken several times and tilted several times to ensure homogenization of the two solutions. The plate was kept inside the incubator and within 4 h, the transfection of the cells was complete. Cells were allowed to grow for 36 h and were assayed for caspase 2 mRNA insert silencing using the Dual-Glo™ Luciferase Assay System from Promega. Initially firefly luciferase emission was recorded and then

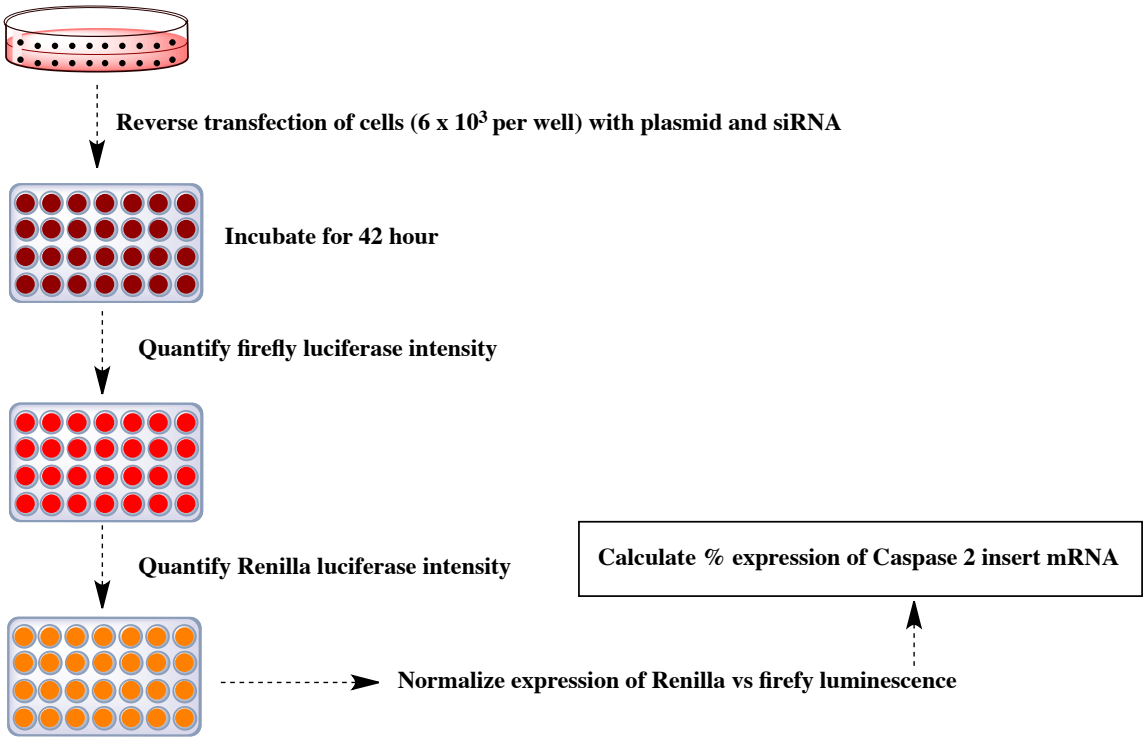


Figure 3.5. RNAi protocol.

by adding the Dual Glo Stop and Glo reagent, the firefly luciferase was terminated and Renilla luminescence was recorded. Ratios of Renilla to firefly luminescence of samples were used to compute percent expression of the caspase 2 mRNA.

The PKR binding assay. This experiment (Figure 3.6) was performed by Erik Fostvedt and Rachel Valenzuela in the laboratory of Professor Peter A. Beal, Department of Chemistry, University of California, Davis. U87 cells (approximately 8 x 10^6 in a 75 cm^2 flask) were treated 24 h before lysis with human interferon-α A (PBL Interferon Source) to a final concentration of 1 x 10^6 U L^{-1}. The interferon-treated cells were washed twice with PBS and lysed by shaking with 3 mL solubilization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, supplemented with protease inhibitor cocktail (ProteoBlock, Fermentas) for 30 min on ice. The lysates were clarified by centrifugation at 14000 x g at 4 °C for 20 min and used directly in pull-down experiments.

Magnetic streptavidin beads (0.5 mg, 50 µL) (Dynabeads M-280, Invitrogen) were prepared for RNA manipulation according to the manufacturer's protocol. Briefly, beads were suspended in 350 µL binding buffer (5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 M NaCl) and coated with 100 pmol of siRNA (100 µL) by gently rocking for 30 min at room temperature. The siRNA-coated beads were washed twice with binding buffer (500 µL) and once with solubilization buffer (500 µL) and incubated with 0.5 mL cell lysate for 20 min at room temperature. Beads without siRNA were also incubated with cell lysate and served as a control. After incubation, the beads were washed four times with 500 µL wash buffer (20 mM HEPES, pH 7.9, 2.5 mM $MgCl_2$, 100 mM KCl, 20% glycerol, 0.5 mL DTT, 0.2 mg/mL yeast RNA, 0.2 mg/mL salmon sperm DNA). Bound protein was eluted from the beads by heating in the loading buffer, separated on 6% SDS-

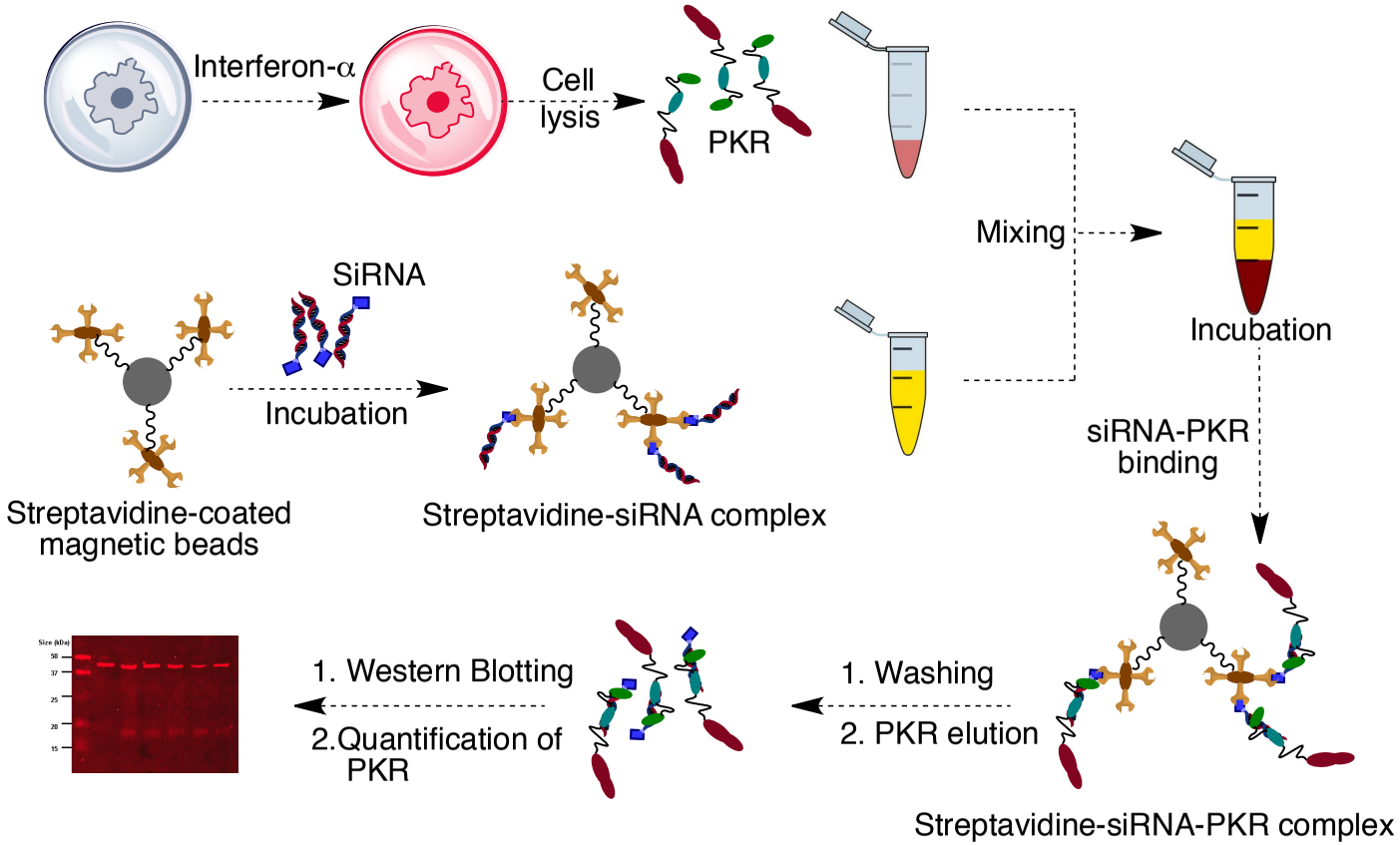


Figure 3.6. PKR binding experiment protocol.

PAGE and transferred to a PVDF membrane for Western blotting. Membranes were blocked with blotting-grade milk (Bio-Rad), incubated with PKR antibody (Santa Cruz Biotechnology, 1:1000 dilution), washed with TBS-Tween, and incubated with alkaline phosphatase-conjugated secondary antibody (Santa Cruz Biotechnology, 1:2000 dilution). The proteins were detected using ECF substrate (GE Healthcare) on a Typhoon Trio Variable Mode Imager (GE Healthcare) and band intensities were quantified using ImageQuant software (Molecular Dynamics). PKR binding affinity is reported as the average ratio of band intensities of modified siRNA to native siRNA for three independent experiments.

Results and discussion

Thermal analysis of the 8-alkoxyA-containing siRNAs. Unmodified and modified (8-alkoxyA) guide strands were annealed to corresponding passenger strands (Tables 3.1 and 3.2) by heating to 90 °C and slowly cooling down to room temperature in 2 h. The guide and passenger strands were designed in such a way that 8-alkoxyA faces G or U in the corresponding passenger strands. The unmodified A:U duplex was used as a reference, based upon which the thermal stabilities of the singly- and doubly-modified duplexes (both 8-ROA:U and 8-ROA:G) were analyzed. In the case of the 8-ROA:U base pair, the 8-ROA nucleoside can adopt the canonical *anti* orientation around the glycosidic bond. For the 8-ROA:G pair, 8-ROA_{syn}:G_{anti} and 8-ROA_{anti}:G_{anti} combinations were considered, while other A:G combinations were deemed too distorting to exist in an A-form duplex RNA. However, the 8-ROA_{anti}:G_{anti} pair will widen the helix diameter considerably and introduce strain into the duplex; additionally, the presence of a significantly large alkoxy group at position 8 of the purine ring favors the 8-ROA_{syn}:G_{anti}

combination. Thus, the existence of 8-ROA$_{anti}$:G$_{anti}$ cannot be ruled out, but 8-ROA$_{syn}$:G$_{anti}$ is more likely to occur in A-form duplex RNA.

All singly and doubly modified duplexes displayed lower melting temperatures (T_m) compared to the unmodified duplex (Figures 3.7 and 3.8). Therefore, the alkoxy group appears to introduce instability in the RNA duplexes. In all duplexes, the 8-ROA:U base pair was found to be more stable than the 8-ROA:G base combination implying that the Watson-Crick hydrogen-bonding is still preferred in these modified adenosines, provided that 8-ROA faces U as the complementary base. Although 8-ROA:G also has a greater potential to expose its Hoogsteen face to *anti* G, steric clash between the alkoxy group of 8-ROA and the exocyclic amine of G in the minor groove might undermine the strength of hydrogen bonding resulting in lower T_m values for 8-ROA:G.

Melting temperatures also varied depending on the position of the 8-alkoxyA base in the guide strand. It is not clear why the 5′-end of the guide strand can tolerate modifications of variable size more effectively than the other end. The 5′-end of the guide strand is thermodynamically less stable and hence more prone to unzipping; introducing a destabilizing nucleoside in this region, as opposed to the other end, should further decrease the thermal stability of the duplex. Again at positions 10 and 15, the 8-alkoxyAs are between two purines, so a greater stacking interaction is expected. These siRNAs would be expected to have higher melting temperatures compared to siRNAs bearing these modifications at positions 4 and 6. In practice however, the reverse trend is observed. In all cases, higher duplex stability was obtained when the modifications are closer to the 5′-end of the guide strand. Modifications in the middle position (10) or towards the 3′-end (15) of the guide strand always exhibited reduced thermal stability.

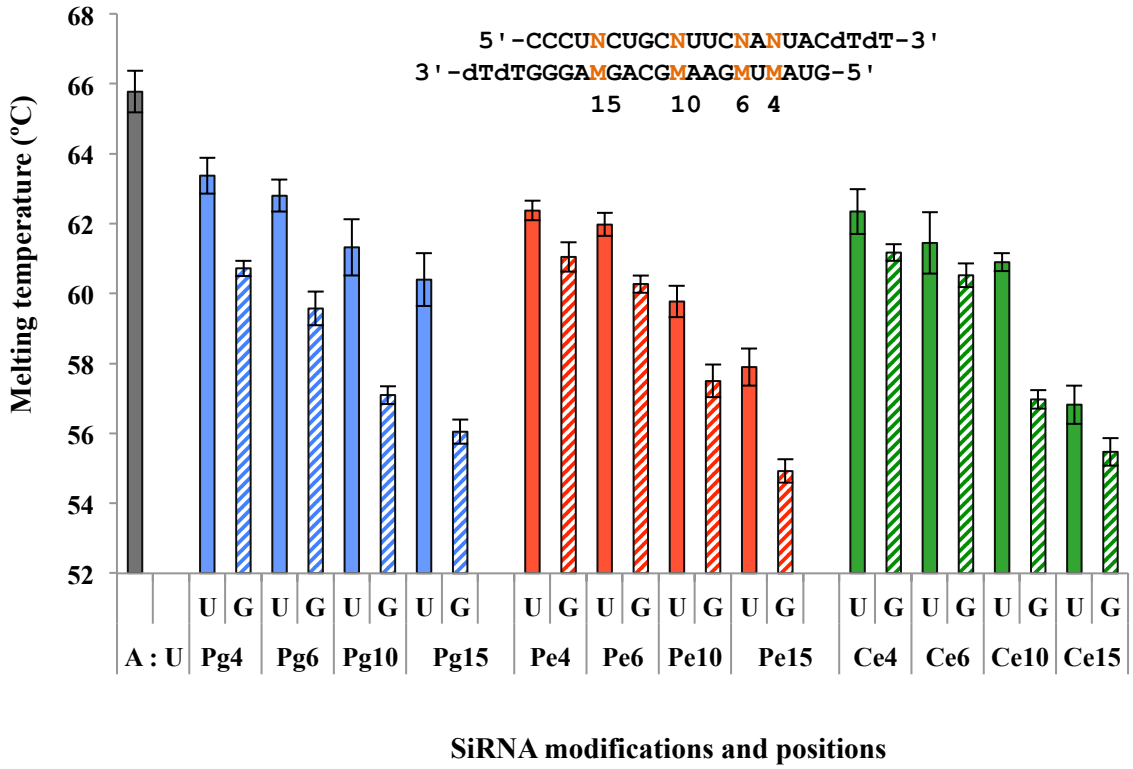


Figure 3.7. Thermal analysis of the siRNA duplexes with single 8-alkoxyA modifications at four different positions, 4, 6, 10 and 15, in the guide strand. Modifications were placed opposite to U or G in the corresponding passenger strands. The sequences are shown in the tables 3.1 and 3.2; here M represents 8-PgOA, 8-PeOA or 8-CeOA, and N is either U or G.

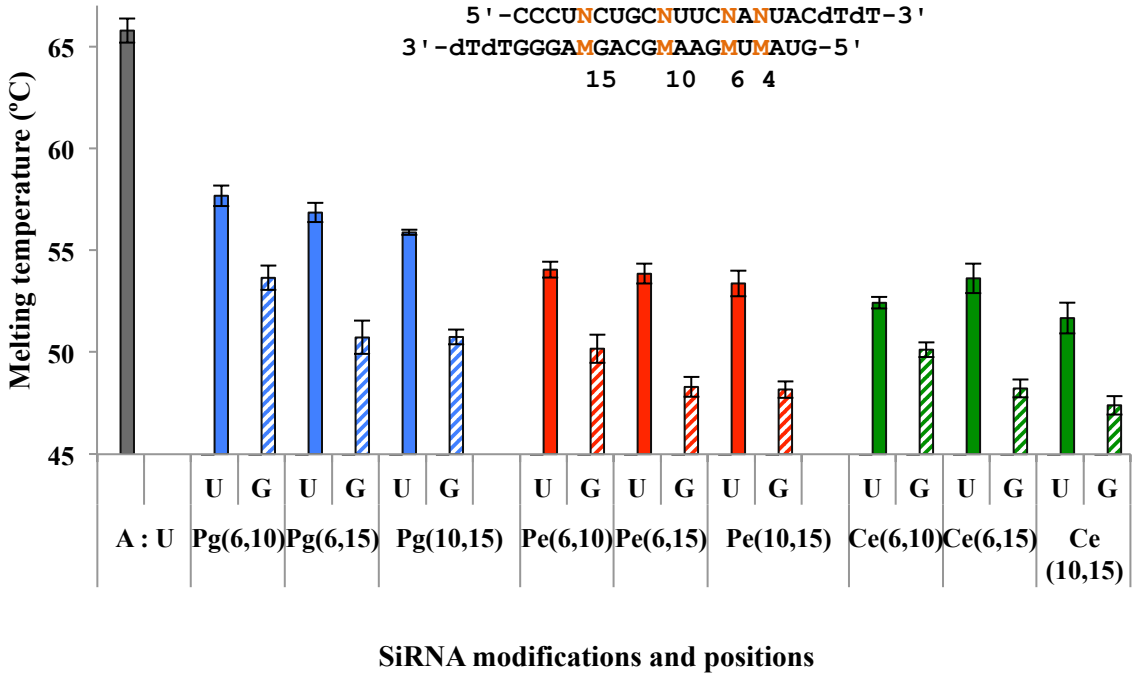


Figure 3.8. Thermal analysis of the siRNA duplexes with double 8-alkoxyA modifications at three different positions, 6, 10 and 15, in the guide strand. Modifications were placed opposite to U or G in the corresponding passenger strands. The sequences are shown in the tables 3.1 and 3.2; here M is 8-PgOA, 8-PeOA or 8-CeOA, and N is either U or G.

For single modifications, lowering of the T_m was moderate for modifications at positions 4 and 6 opposite U (e.g., Pg4:U, Pg6:U, Ce4:U); however single modifications at positions 10 and 15 exhibited significantly lower T_m irrespective of their placement opposite to G or U. As expected, reduction in duplex stability was more pronounced (7-14 °C) for doubly modified duplexes. Interestingly, except for position 15, the size of the alkoxy groups did not have much influence on the thermal stability of siRNA duplexes.

A similar trend was noticed in 8-ClA-containing RNA duplexes which also have lower melting temperatures compared to the unmodified duplexes (*56*). Therefore, thermal destabilization cannot be primarily linked to the substituent size and shape effect, but also to substituent electronegativity values. Because both Cl and O have higher electronegativities compared to H, a strong electron withdrawing inductive effect may operate to reduce electron density in the purine rings significantly, and hence this can reduce the stability of hydrogen bonds formed by 8-ClA:U or 8-ROA:U in the Watson-Crick base pairing and affect base stacking as well.

Lowering of the T_m in the 8ROA:U base pair compared with the A:U base pair can also be explained in terms of a relatively pronounced *syn* preference of 8-substituted purines over the *anti* conformation, both in the context of DNA (*57, 58*) and RNA (*56, 59-61*) nucleosides and duplex oligonucleotides. X-ray crystallographic (*59*) and CD studies (*60*) of 8BrA and the corresponding 5′-monophosphate have shown that these molecules predominantly exist in the *syn* conformation around the glycosidic bond due to the presence of a sterically demanding Br atom at position 8 in the purine ring. Although the van der Waals radius of Cl (1.80 Å) is significantly smaller than Br (1.95 Å), theoretical calculations predicted preferentially the *syn* conformation even for 8-ClA

nucleosides (*61*). Importantly, a relative lowering of melting temperature of A:U vs. A:G combination was noticeably greater than the 8-ROA:U vs. 8ROA:G combinations. Interestingly, this difference shrank even more for the 8-PeOA and 8-CeOA containing duplexes, implying that with increasing the steric bulk of the alkoxy group, the *syn* preference of the 8-alkoxyadenosines increased. All these observations fit well with the preferential *syn* conformation of the 8-substituted-adenosines, such as 8-ClA and 8-ROA, in the duplex RNA context.

Caspase 2 mRNA knockdown studies with the modified siRNAs. To analyze caspase 2 expression levels quickly and reliably, a plasmid-based dual luciferase assay system was employed. A fragment of the caspase 2 mRNA sequence was inserted into the psiCHECK2 vector, and the resulting reporter plasmid was used to evaluate caspase 2 mRNA knockdown.

Singly substituted siRNAs, alkoxylated at several positions of the guide strand, exhibited almost equivalent or slightly lower silencing effects compared to the unmodified caspase 2 siRNA (Figure 3.9). Among singly modified siRNAs, 8-PgOA exhibited the highest mRNA knockdown efficiency. Surprisingly, 8-CeOA modifications, even though the largest among three modifications, showed almost equal knockdown efficiency to 8-PgOA, whereas, 8-PeOA containing siRNAs were found to have the lowest efficacy. Thus, the mRNA knockdown ability of the modified caspase 2 siRNAs does not directly correlate with the substituent size.

Interestingly, singly modified siRNAs with intermediate thermal stability (modifications at positions 6 and 10) exhibited higher RNAi efficacy than those having the highest (modification at position 4) or the lowest (modification at position 15)

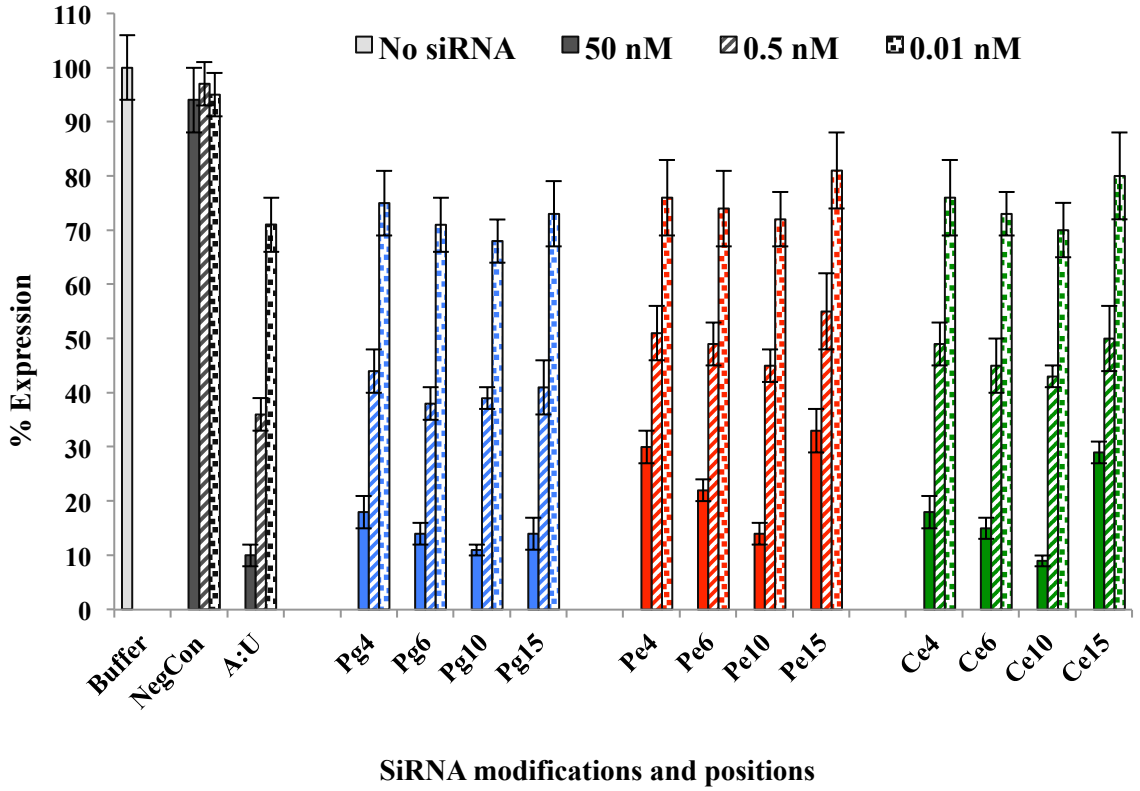


Figure 3.9. RNAi studies with the singly modified siRNAs. % Expression of Renilla luciferase relative to firefly luciferase when treated with siRNAs bearing single 8-alkoxyA modification at position 4, 6, 10 or 15 in the guide strand. 8-AlkoxyA (8-PgOA, 8-PeOA or 8-CeOA) modifications were placed opposite to G (in the passenger strand) during delivery and U (in the mRNA) in the RISC. The siRNA sequences are depicted in Table 3.2. Experiments were conducted at three different concentrations: 50 nM, 0.5 nM and 0.01 nM.

stability (Figure 3.9). This observation supports the basic rules of siRNA design and efficacy, which emphasizes that, for optimal efficacy, the thermal stability of the siRNA duplexes be intermediate, neither very high, nor very low (*62, 63*). If the stability is too high then RISC will be unable to unzip the siRNA duplexes, and hence mRNA knockdown efficiency will be lowered. On the other hand, if the duplex stability is low then the effective concentration of double-stranded siRNAs will be lowered and RNAi efficacy will be drastically reduced. Because the differences in T_m among the four sequence contexts is not very high, their influence on the RNAi efficacy engenders curiosity.

In all cases, modifications at position 10 of the guide strand exhibited the highest gene-silencing efficacy, followed by position 6. Modifications at position 15 seemed to reduce the potency of siRNAs, with the exception of the propargyloxy modification, which was found to be equally active when compared to other positions in the series. So, position 15 was found to tolerate smaller groups better as opposed to larger groups such as phenylethoxy and cyclohexylethoxy groups. This trend is somewhat unexpected, since it is already known that substitutions towards the 3′-end of the guide strand typically can tolerate chemical modifications and even mismatches rather well (*11, 38*). Contrarily, the seed region and the cleavage site are more sensitive to chemical modifications and mismatches, in general. Sometimes, a single mismatch (*62*) at the center or chemical modifications distorting the A-form duplex structure (*6*) abolishes the efficacy of the corresponding siRNA. In this case, modifications in two important positions of the seed region with large substituents are quite well tolerated. More surprisingly, a large modification adjacent the cleavage site (position10) actually rendered the siRNA more

effective towards knocking down caspase 2 mRNA. This trend was observed for all modifications; however with larger modifications such as 8CeOA and 8PeOA, this trend was observed without any ambiguity. The presence of a hydrophobic pocket at the corresponding position in Argonaute2 in the RISC might be a plausible explanation for this observation (*64*).

Multiple 8-ROA substitutions at the guide strands showed significantly reduced silencing efficacy compared to their corresponding positive control siRNAs or single modifications (Figure 3.10). This trend holds irrespective of the substituent size, shape and position. These siRNAs only exhibit significant mRNA knock down at 50 nM concentrations.

Modified purines at positions 6 and 10 in the guide strand opposite to G or U (in the passenger strand) did not substantially change the knock down efficiency (Figure 3.11). In most of the cases, delivering the modified purine opposite to G brought about almost equal or slightly higher mRNA knockdown than delivering it opposite to U. Only with Ce6, a reverse trend was observed, though it was only by a small margin. These data suggest that both 8-ROA:G and 8-ROA:U can be loaded into the RISC with equal efficiency.

Importance of Switching the Steric Blockade from the Minor to the Major Groove in the RISC. To explore the importance of 'base switching' in the context of RISC-mediated cleavage of caspase 2 mRNA, two mutant plasmids, P6 and P10 were synthesized using appropriate inserts by mutating specific positions of the wild-type caspase 2 insert. Two important sites in the guide strand of the caspase 2 siRNA, positions 6 (in the seed region) and 10 (adjacent the cleavage site) were chosen for the

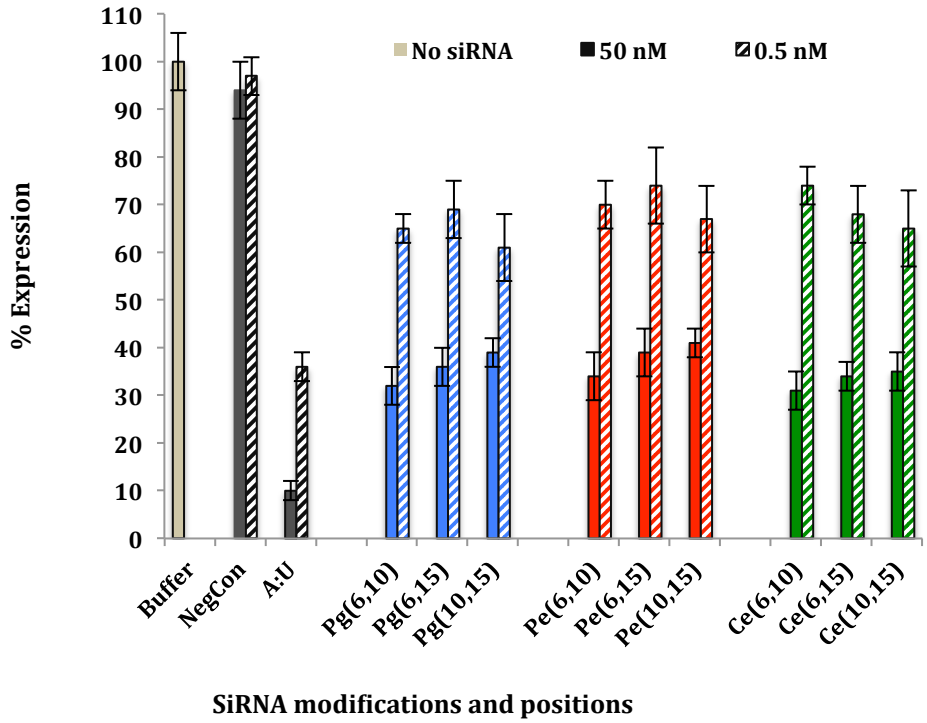


Figure 3.10. RNAi studies with the doubly modified siRNAs. % Expression of the Renilla luciferase relative to the firefly luciferase when treated with siRNAs bearing double 8-alkoxyA (8-PgOA, 8-PeOA or 8-CeOA) modifications in three different combinations in the guide strand: (6, 10), (6, 15) and (10, 15). 8-AlkoxyA modifications were placed opposite to G in the passenger strand during delivery and U (in mRNA) in the RISC. The siRNA sequences are depicted in Table 3.2. Experiments were conducted at two different concentrations: 50 nM and 0.5 nM.

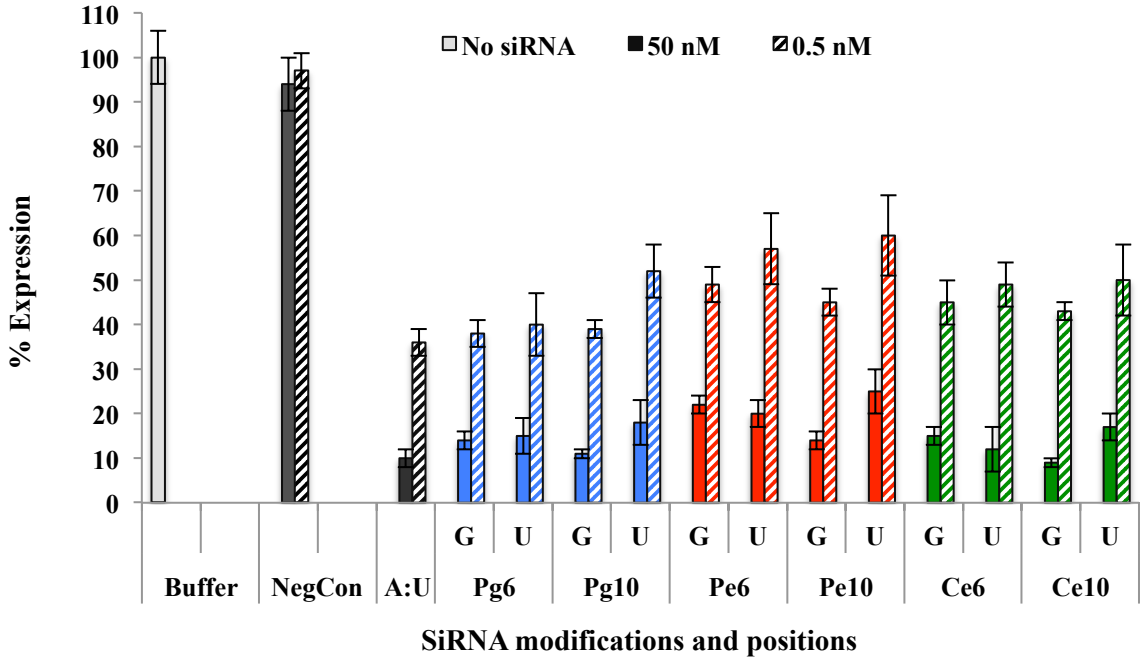


Figure 3.11. % Expression of the Renilla luciferase relative to the firefly luciferase when treated with siRNAs bearing 8-alkoxyA (8-PgOA, 8-PeOA or 8-CeOA) modifications at positions 6 and 10 opposite to either G or U.

construction of mutant plasmids, P6 and P10. In such cases, the 8-alkoxyAs face G as the complementary base both during the siRNA delivery and during interaction of the guide strand with the mRNA in the RISC. Therefore, alkoxy steric blockades will be presented in the minor groove both in the guide-passenger and guide-mRNA constructs.

As expected, the mRNA knockdown efficiency was drastically reduced for all three (8-PgOA 8-PeOA and 8-CeOA) modifications; 8-PgOA being the smallest in size is relatively more efficacious (Figures 3.12 and 3.13). In the case of A:G mismatched guide SiRNA:mRNA constructs, a similar decrease in mRNA knockdown efficiency was noticed with respect to both mutant plasmids, P6 and P10.

These results indicate that for the guide:mRNA duplex, both local widening (*65*) (due to the A:G pair) or minor groove crowding (due to the 8ROA:G pair) can lead to drastic reduction of the mRNA knockdown efficacy. Probably these distortions in the guide:mRNA duplex do not allow the necessary protein side chain interactions in the guide:mRNA:RISC ternary complex.

The PKR binding studies. To assess the effect of these modifications on siRNA binding to an off-pathway dsRNA-binding protein, we used a PKR binding assay involving immobilized biotinylated siRNA (*11*). These experiments were conducted by Erik Fostvedt and Rachel Valenzuela in the laboratory of Prof. Peter A. Beal, Department of Chemistry, University of California, Davis. 5′-Biotinylated passenger strands with either U or G opposite to the modified purines were annealed to Pg6, Ce6, Pe6, Pg10, Ce10, Pe10 and unmodified guide strands. The biotinylated duplexes were attached to magnetic streptavidin beads and used in affinity purification for PKR. The degree of PKR binding for each duplex was then analyzed by western blot.

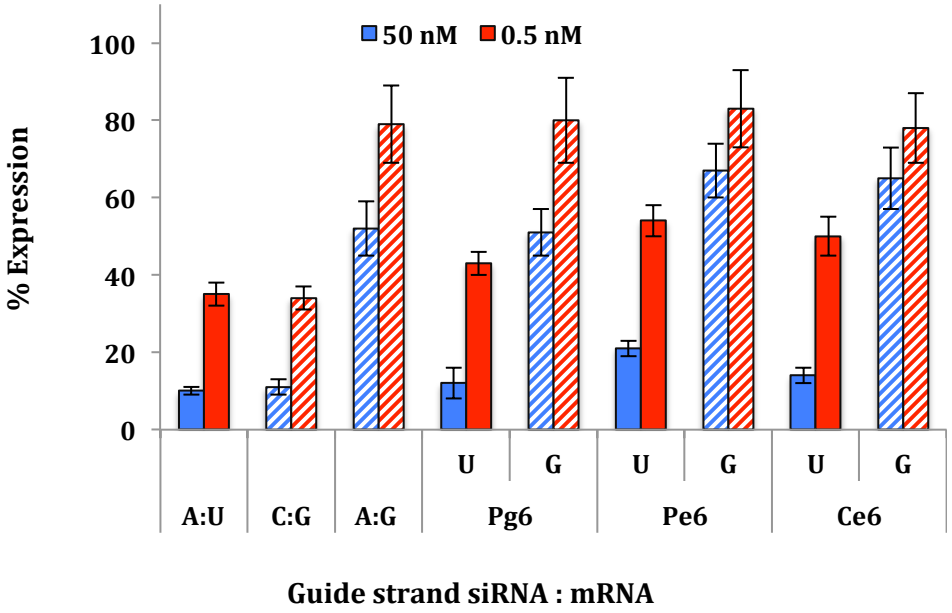


Figure 3.12. RNAi studies on the P6 mutant plasmid (caspase 2 insert containing plasmid mutated at position 6 corresponding to the guide siRNA). % Expression of the Renilla luciferase relative to the firefly luciferase, when treated with siRNAs bearing modifications at position 6 in the guide strand, and targeted U (from A6:U plasmid, depicted by solid bars) vs. G (from C6:G plasmid, depicted by hashed bars) in the caspase 2 insert and mutant caspase 2 insert mRNA respectively. Sequences are given in Tables 3.1 and 3.2. Experiments were conducted at two different concentrations: 50 nM and 0.5 nM. A:U, Pg6:U, Ce6:U and Pe6:U represents targeting of U in the caspase 2 insert mRNA (from A6:U plasmid) and C:G, A6:G, Pg6:G, Ce6:G and Pe6:G represents targeting of G in the mutant mRNA where a U was mutated to G (from C6:G plasmid). Nomenclature of the plasmid comes from corresponding modification position in the guide strand of caspase 2 insert mRNA.

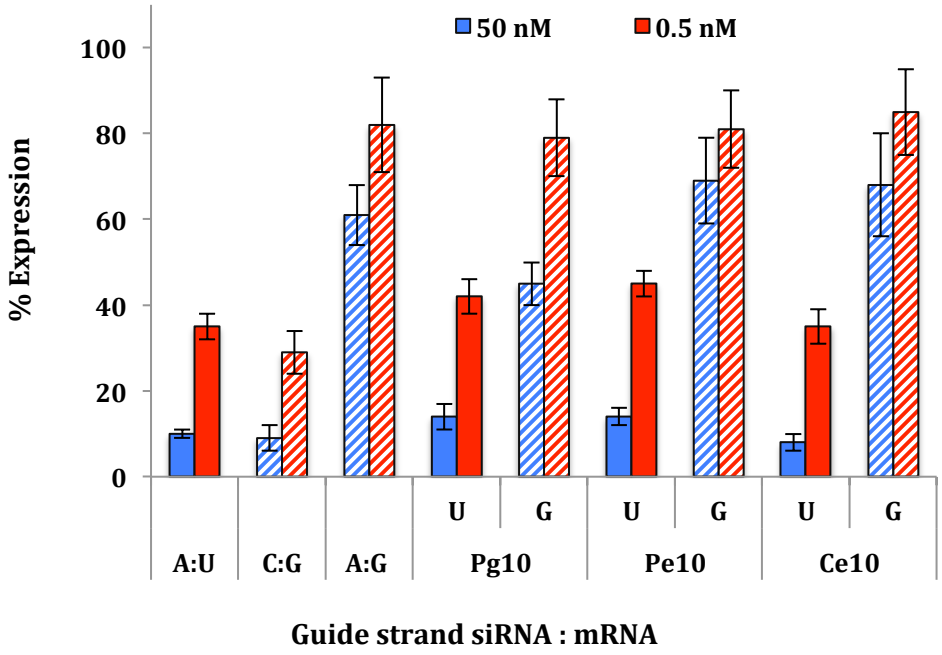
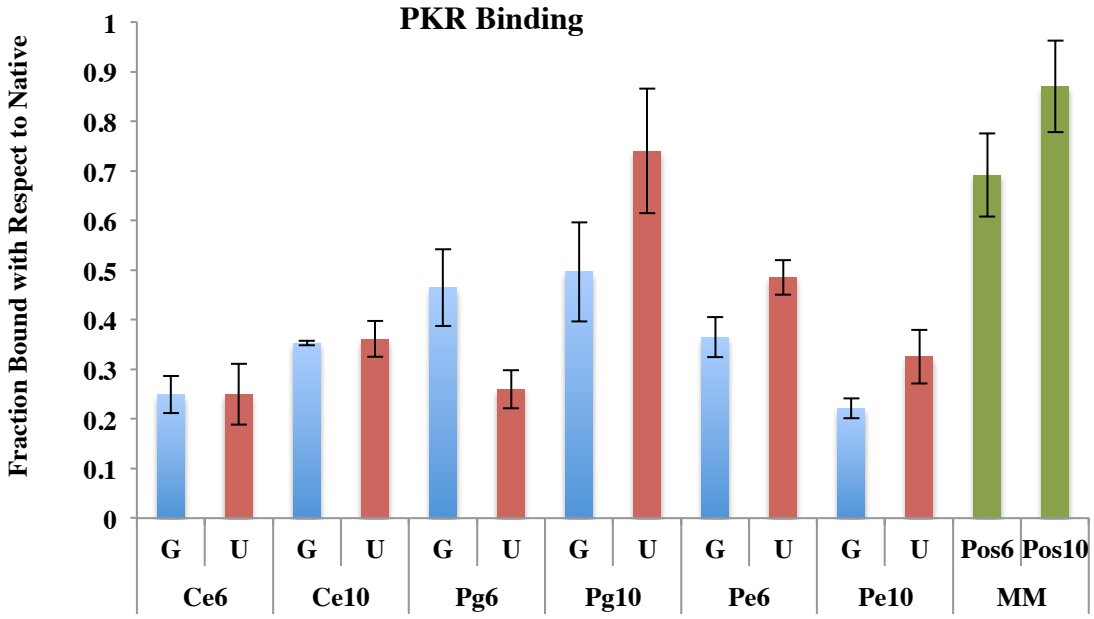


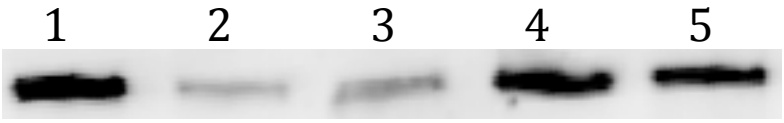
Figure 3.13. RNAi studies on the P10 mutant plasmid (caspagse 2 insert containing plasmid mutated at position 10 corresponding to the guide siRNA). % Expression of the Renilla luciferase relative to the firefly luciferase, when treated with siRNAs bearing modifications at position 10 in the guide strand, and targeted U (from A10:U plasmid, depicted by solid bars) vs. G (from C10:G plasmid, depicted by hashed bars) in the caspase 2 insert and mutant caspase 2 insert mRNA, respectively. Sequences are given in Tables 3.1 and 3.2. Experiments were conducted at two different concentrations: 50 nM and 0.5 nM. A:U, Pg10:U, Ce10:U and Pe10:U represent targeting of U in the caspase 2 insert mRNA (from A6:U plasmid) and C:G, A10:G, Pg10:G, Ce10:G and Pe10:G represent targeting of G in the mutant mRNA where a U was mutated to G (from C10:G plasmid). Nomenclature of the plasmid comes from corresponding modification position in the guide strand of caspase 2 insert mRNA.

Our results show that different types of modifications, when placed in the guide strand opposite to G or U in the passenger strand, are significantly less prone to bind to PKR compared to the unmodified sequences (Figures 3.14 and 3.15). Unmodified siRNAs with A:G mismatches at positions 6 and 10 are almost as susceptible to bind PKRs as the unmodified one. So, prevention of protein binding is not a result of mere bulges due to A:G mismatches, but rather an outcome of the placement of different steric blockades in the minor and major groove by means of placing 8-alkoxyA opposite to U or G.

With propargyloxy modifications being the smallest in size of those studied here, Pg6:G, Pg10:U and Pg10:G P were the least effective in preventing PKR binding with the exception of Pg6:U; whereas all cyclohexylethoxy and phenylethoxy modifications reduced the PKR binding significantly. It seems that PKR is slightly more prone to bind to modified siRNAs where U in the passenger strand was placed opposite to the modification in the guide strand than to siRNAs where modifications are opposite to G. In some cases (for example, Pg10:U vs. Pg10:G, Pe10:U vs. Pe10:G) this discrimination is more pronounced, and in some cases (Pg6:U vs. Pg6:G, Pe6:U vs. Pe6:G) less. These data suggest that this type of steric occlusion of the minor groove is moderately or marginally advantageous over the major groove occlusion. Surprisingly, when 8-CeO modifications were placed in position 6 and 10 opposite to U or G, no distinction was observed between the binding affinities towards PKR. Both Ce6:G and Ce6:U were equally effective in preventing PKR binding. A similar trend with lesser efficacy was observed with Ce10:G and Ce10:U.



A.



B.

Figure 3.14. PKR binding to the modified siRNAs containing 8-alkoxyadenosine 'switches'. (A) Biotinylated siRNAs were bound to magnetic streptavidin beads and treated with lysates from U87 cells treated with IFN-α. The amount of PKR retained was determined by western blotting. Cyclohexylethoxy (Ce), phenylethoxy (Pe), and propargyloxy (Pg) modifications were tested at positions 6 and 10 opposite either G (Blue) or U (Red). Strands with A:G mismatches were also tested at positions 6 and 10 as controls (Green). (B). A representative western blotting consisting of 1. Unmodified siRNA; 2. PgG6; 3. PgU6; and 4,5. MMPos6 in duplicate, here MM refers to mismatch.

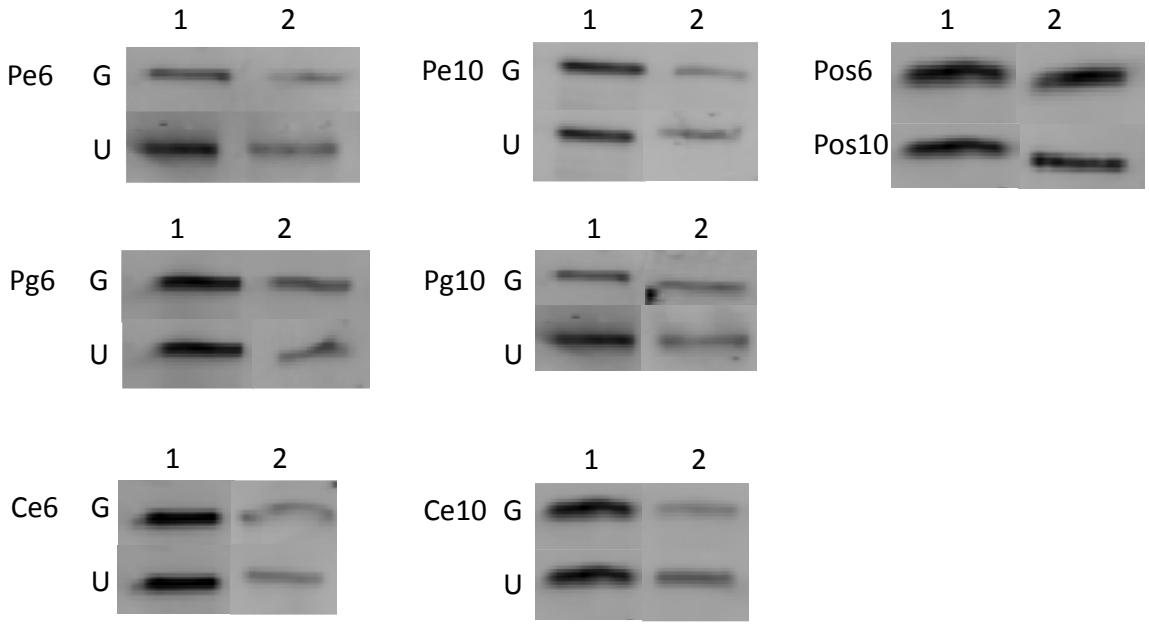


Figure 3.15. Typical western blot results for each of the duplexes tested for off-pathway protein-binding. Each modified strand (column 2) presented here is in comparison to the unmodified duplex (column 1)

There are other reports of prevention of PKR binding by manipulating minor groove modifications (*10, 11, 39*); however, there is no report, so far, of any major groove modification that is equally efficient in attenuating siRNA-PKR interaction when placed in the same position of an siRNA. 8-PeOA and 8-CeOA modified siRNAs are the first examples to exhibit such a characteristic. From the thermal analysis data and RNAi studies with mutant plasmids, it is clear that 8-ROA:U and 8:ROA:G combinations are distinct, their differences likely related to different glycosidic bond conformations and placement of the steric blockades.

Kannan et al. reported N^2-alkylated 2′-deoxy-7,8-dihydro-8-oxoguanosine-based switches are capable of preventing PKR binding only when the steric blockade is placed in the minor groove as opposed to the major groove (*39*). In that study N^2 -propyl and N^2-benzyl modified 8-oxoguanines were used. The phenylethoxy and cyclohexylethoxy groups used here are not only larger, but also significantly longer than the propyl and benzyl groups used in the previous studies. Additionally, substitution of the position 8 of purines with bulky alkoxy groups might significantly affect the overall structure of the major and the minor groove, which might also contribute towards prevention of the PKR binding. Hence, irrespective of their position in either groove, these larger and longer groups repel PKRs to a much greater extent.

Another probable rationale for the high activity of 8ROA:U might be the modification site (position 8) of adenosine itself. PKR is known to bind dsRNA by interacting with two segments of the minor groove and the intervening major groove (*66*). In the major groove, lysine side chains of PKR interact with the negatively charged phosphate backbone of dsRNA. Hence, appropriate major groove modifications of the

siRNA can potentially prevent this electrostatic interaction and block PKR binding. In the case of 8ROA$_{anti}$:U-containing siRNAs, the alkoxy group is suitably positioned to disrupt such major groove interactions with the siRNA.

Conclusion

A general solution to sequence-independent off-pathway protein binding of siRNAs, while maintaining mRNA knock down efficacy, was investigated here. The study also explored the scope of guide strand modifications by utilizing 8-alkoxyadenosines of variable alkyl group size. This is an unusual example in which a purine ribonucleoside is proposed to exist in both *syn* and *anti* conformations around the glycosidic bond depending on the base-pairing partner. 8-Alkoxyadenosine modifications were tolerated both in singly and doubly modified duplexes irrespective of G or U as the pairing partner, although lowering of the T_m is very pronounced in cases of multiple modifications. Singly modified siRNAs were almost the same or slightly less effective in RNAi, whereas multiply modified siRNAs exhibited much lower caspase 2 insert mRNA knock down activity. Singly modified siRNAs were capable of inhibiting PKR-siRNA interactions compared to the unmodified siRNA. The necessity of 'base switching' in the RISC was demonstrated by using two caspase 2 inserts, individually mutated at positions 6 and 10. To confirm the *syn* vs. *anti* conformational preference of 8-substituted adenosines in appropriate duplex RNA contexts, NMR spectroscopic studies are currently in progress.

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CHAPTER 4

CONCLUSION AND FUTURE DIRECTIONS

Numerous chemical modifications have been implemented to make siRNA therapeutically more potent and selective. In addition to the phosphate backbone and the ribose modifications, several successful base modifications have also been reported. Modifying passenger strand bases is more difficult compared to other modifications, and it is extremely challenging to modify guide strand bases. However, base modifications of the siRNA can help explore the effect of H-bonding and sterics in the context of RNAi machinery. In this study, 8-alkoxyadenosines were introduced into the guide strands and their effects on the RNAi efficacy and the off-pathway protein binding were explored.

8-Substituted purines are known to exist in an equilibrium mixture of *syn*/*anti* conformations. Hence, here 8-alkoxyadenosines were used as a conformational 'switch' to mediate RNAi and alleviate off-pathway protein binding efficiently. 8-AlkoxyA phosphoramidites were synthesized and incorporated into the guide strand of a caspase 2 siRNA. Alkoxy groups were chosen based on their sizes and shapes.

All the singly and doubly modified guide strands formed stable duplexes with appropriate passenger strands, although the thermal stability of the siRNAs gradually decreased with each substitution. The thermal stability of the duplex oligoribonucleotides did not depend too much on the size of the alkoxy group. Electron withdrawing inductive

effect of O is thought to weaken the hydrogen bonding between the modified nucleoside and the complementary base, resulting in lower T_m values for the modified siRNAs.

The singly modified siRNAs have RNAi efficacy similar to the unmodified positive control; but double substitutions of 8-alkoxyA in the guide strand significantly lowers the RNAi efficacy. All 8-alkoxyA modifications were tolerated in the seed region and at the cleavage site of the siRNA. The phenylethoxy modification was found to be the least effective among all modifications.

Modifications at positions 6 and 10, when placed opposite to U or G, could prevent the off-pathway interaction of siRNA with the PKR. Placement of the steric blockade in the minor groove (8-alkoxyA:G) was effective in preventing PKR binding; surprisingly steric blockades, when placed in the major groove (8-alkoxyA:U) were also effective, although to a lower extent than the earlier mode of placement, in knocking down the siRNA-PKR interaction.

From the T_m analysis, RNAi studies and PKR binding experiments, it can be inferred that 8-alkoxyAs have the potential to exist in both *syn* and *anti* conformation depending on the base-pairing partner. However, 'switching' of the 8-alkoxyadenosine nucleosides can be explored comprehensively and unambiguously only through direct structural analysis of the duplex RNAs containing these lesions. Both X-ray crystallography and NMR spectroscopy can be used to explore the base switching, but x-ray crystallographic analysis of the duplex RNA is extremely difficult since crystalizing RNA is not trivial. Therefore NMR spectroscopy seems to be a practical choice for this purpose.

Structural analysis of double-stranded RNA and DNA employing 1D and 2D NMR spectroscopic methods is a routine procedure these days; however most of the previous studies involved natural bases. In exploring hydrogen bonding between bases, 1D immino region spectrum and 2D nuclear Overhauser effect spectroscopy (NOESY) are valuable techniques and will be employed here.

Initially, a 14-mer self-complementary RNA duplex (Figure 4.1) of reasonable thermal stability was taken as the reference strand. 8-Propargyloxyadenosine was chosen as a representative modification to explore the switching probability of the 8-alkoxyadenosines. Initially, we wanted to explore the switching mechanism with nucleosides such as 8-BrdA or 8-PrNHdA, which are known to exist in both *syn* and *anti* conformation depending on the base-pairing partner. Also, substituting a single ribonucleotide with a deoxyribonucleotide does not affect the helical geometry of the double-stranded RNA – it is still an A form helix. Locating different conformers of a deoxyribonucleotide will be easier in the 2D NMR of a duplex RNA and, thus, can serve as the starting point in this structure elucidation project. 8-BrdA was found to be acid-labile and therefore we designed strands containing 8-PrNHdA; later 8-PrgOA would be used in place of 8-PrNHdA.

To summarize, an unusual base modification, 8-alkoxyA, in the guide strand was found to be functional in mediating RNA interference and preventing off-pathway PKR binding with the siRNA in cultured mammalian cells. The next step would be to evaluate efficacy of these modifications in animal models. These of modifications also hold promise in preventing sequence-specific binding of siRNAs with TLR7 leading to immu-

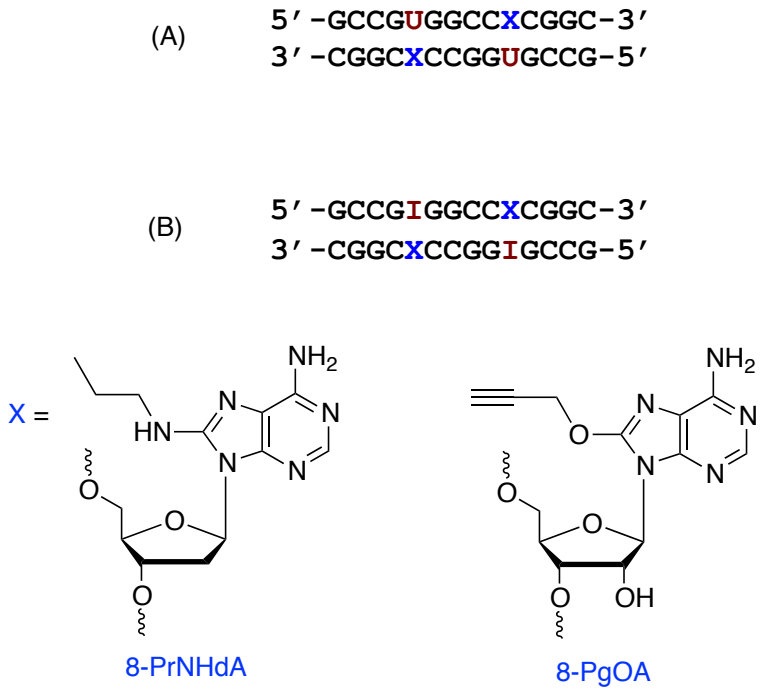

Figure 4.1. Self-complementary 14-mer RNA duplexes depicting **(A)** 8-PrNHdA:U and 8-PgOA:U combinations and **(B)** 8-PrNHdA:I and 8-PgOA:I combinations.

nostimulation. Similar modifications with added properties, such as fluorescence, might help explore details of the RNAi mechanism.

Structural analysis of 8-PrNHdA containing self-complementary duplex RNAs is currently under investigation. Each duplex contains two 8-PrNHdA nucleotides in two strands and U or I opposite to these modified bases. Our hypothesis is that 8-PrNHdA will adopt the *syn* conformation when placed opposite I, whereas it will adopt an *anti* conformation when it faces U in the complementary strand. The structural features of different conformations will be explored through 1D imino region ^{1}H NMR and 2D NOESY. Similar experiments will be conducted with 8-PrgOA in place of 8-PrNHdA to elucidate the 'base-switching' hypothesis related to 8-alkoxyadenosines.